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The Role of the EAR Domain in Transcriptional Repressors involved in Plant Defence

This thesis is submitted for the degree of MPhil by research to the University of Exeter

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Submitted by Felicity Emma George, to the University of Exeter as a thesis for the degree of Master of Philosophy by Research in Biological Sciences, May 2019.

I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

(Signature) ……

Abstract

Plants respond to abiotic and biotic stressors through hormone signalling which forms an integral part of the plant immune response, and is often the target of phytopathogens. Changes in hormone levels are often underpinned by transcriptional reprogramming driven by specific **t**ranscription **f**actors (TF) which collectively regulate the spatial, temporal expression levels of hormone biosynthetic and signalling genes in a co-ordinated manner.

This study examined two contrasting **t**ranscription **r**epressors (TR) implicated in *Arabidopsis* - *Pseudomonas syringae* disease development; a MYB like transcription factor (HUB37) and a **JA**smonate **Z**im domain (JAZ) containing transcriptional repressors involved in jasmonate signalling. The MYB transcription factor was identified by modelling as a core hub in immune signalling, whereas JAZ5 was recently shown to co-operate with JAZ10 during transcriptional reprogramming, to restrict *P. syringae* growth and attenuated chlorosis.

The transcriptional repressor **E**RF-associated **A**mphiphilic **R**epression (EAR) domains confer dominant transcriptional repressive functions. HUB37 contains one EAR domain and JAZ5 contains 2 EAR domains.

Previous transcriptional inference modelling predicted HUB37 was a highly transcription factor that negatively regulated *A. thaliana* defence responses to *P. syringae*. This was validated by testing a HUB37 loss of function mutant *(Siddharth Jayaraman, Marta de Torres per com).* JAZ5 and JAZ10 are required for full immunity to *P. syringae.* Thus, this study sought to develop molecular and genetic tools to explore the role of the EAR domain in disease.

Golden Gate cloning and targeted mutagenesis were used to generate epitope tagged lines of JAZ5 and HUB37 with and without EAR domains. These were assembled into T-DNA transformation vectors and various transgenic lines characterised. At the end of the project we had generated a range of lines and shown that HUB37 was the target for post-transcriptional degradation by bacterial effects. Our data predicts that bacterial effectors function to remove a negative regulator of plant immunity to promote disease.

Acknowledgements

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Genna Davies and Clara Rodriguez Herrero for their complementary tenacious work ethic and continued friendship.

My family for supporting the difficult decision not to relocate with the team. To conclude my research and pass on the tools with scope to the next Scientist was daunting. I am happy in the knowledge that this work is ongoing and I have added to scientific research.

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List of Abbreviations

ABA abscisic acid

bHLH basic-Helix-Loop-Helix

bp base pairs

BR brassinosteriods

dpi days post infiltration

DC3000 *Pseudomonas syringae pathovar tomato* DC3000

COR coronatine

CKs cytokinins

EAR ERF-associated amphiphilic repression

ET ethylene

ETI effector triggered immunity

ETS effector triggered susceptibility

FP forward primer

GA gibberellins

hpi hours post infection

HR hypersensitive response

hrp hypersensitive response and pathogenesis

HUB37 - AT5G47390

JA jasmonic acid

JA-Ile jasmonic isoleucine

JAZ jasmonate – ZIM domain

KO knock-out

L0 level zero

L1 level one

L2 level two

MAMP microbial-associated molecular pattern

NINJA novel interactor of JAZ

PAMPs pathogen associated molecular patterns

PCR polymerase chain reaction

P1 position one

P2 position two

PRR pattern recognition receptor

PTI PAMP triggered immunity

RD repressor domain

RP reverse primer

SA salicylic acid

TFs transcription factors

TR transcriptional repressor

TPL topless

TU transcriptional unit

T3SS type III-secretion system

ZIM zinc finger protein expressed in inflorescence meristem

1. Introduction

Aims and objectives

Hypothesis

Only very recently has it been recognised that transcriptional repression and the removal of negative regulators provide the capacity for a rapid and robust response to stresses. Here, two lines of research were undertaken to examine the roles of two EAR domain containing repressors in the defence responses of *Arabidopsis thaliana* to the hemibiotrophic pathogen, *P. syringae* pv. *tomato* strain DC3000. HUB37 is a MYB transcription factor that directly binds DNA. JAZ5 contains two EAR domains and targets transcription factors involved in jasmonate signalling.

AT5G47390 (HUB37) - is hypothesised to be a target of DC3000 effector proteins and/or part of effector-triggered immunity based on transcript modelling. Knockout lines of *HUB37* are more resistant to DC3000. The protein level of HUB37 is yet to be quantified in response to infection with virulent and non-virulent DC3000. HUB37 has a conserved EAR domain. It is believed that the EAR motif is the active repressive domain of HUB37 interacting with other transcription factors. It is hypothesised that EAR mutated lines are therefore expected to act like *hub37*.

AT1G17380 (JAZ5) – has conserved domains which include two EAR domains. It is hypothesised that these two EAR domains contribute to JAZ5's function as a dominant transcriptional repressor. This study seeks to determine the role of these JAZ5 EAR domains in plant immunity and JAZ5-JAZ10 interactions through generating targeted mutations in the JAZ5 EAR domains.

To enable molecular dissection of the role of EAR domains, constructs were generated to mutate EAR domains and epitope tag wild type and mutated JAZ/HUB37 lines to characterise the role of these TRs in regulating plant immunity.

The contribution made by the thesis in the context of the approved field of study

This thesis addresses a neglected area of plant-pathogen interactions, that is, the role of EAR domains in regulation of plant defence. Overall this research will help increase our knowledge of jasmonate signalling and illustrate the utility of systems biology approaches.

It will generate new resources including *A. thaliana* JAZ5 with a combination of EAR domains mutated with epitope tags. This can be used to identify *in planta* expression and screen yeast 2 hybrid libraries.

Finally, it will characterise a novel MYB transcriptional repressor, which to date has not been implicated in plant defence responses.

Literature Review and Background Studies Underpinning this Study.

Plants are the source of organic carbon to almost all non-photosynthetic organisms on earth. They are sessile organisms but live in complex environments and agricultural productivity is affected by biotic and abiotic interactions. Plants have evolved sophisticated mechanisms to respond to microbe attack. The ability of pathogens to overcome the defence mechanisms of plants usually involves rapid evolution via natural selection for beneficial mutations within the pathogen population. As phytopathogens reproduce rapidly they can evolve to overcome plant immunity, despite concerted breeding efforts and often cause massive crop losses (Agrios, 2005). Such crop losses are exacerbated in many areas where elite varieties are monocultures of genetically identical plants and are hence susceptible to attack by pathogens and bacteria that have rapidly evolved to overcome the host resistance (Smith *et al*., 2010). Climate change, along with an increased demand on resources from a growing population, requires a greater understanding of plant microbe interactions. Today, crop losses through disease is one of the most significant factors impacting food security in both developing and developed countries. Population increase, ~ 9 billion by 2050, means that higher crop yields are required. Significant investment is required to develop new and innovative approaches to improving crops while limiting agrochemical use (Tomlinson, 2013). Regular spraying of crops with chemicals brings both financial and environmental concerns. If farmers are to be successful, they need access to disease resistant crops and have the appropriate biological and chemical measures to protect them.

Plants are normally resistant to most microbes. This resistance may be complete but can vary from partial immunity to complete susceptibility. Physical barriers like surface waxes and pattern recognition receptors on the plant cell surface provide the first level of immune protection. The latter recognise specific conserved molecules on the microorganism and this causes the activation of plant defences known as the basal defence mechanism. These defences are also known as **p**athogen-**a**ssociated **m**olecular **p**atterns (PAMPs) or PAMP-

triggered immunity (PTI; Janeway, 1989; Dangl & Jones, 2006). Ideally, PTI operate at the point of entry and will prevent ingress, reproduction and spread of the disease throughout the plant. Localisation of the pathogen is imperative in the resistance of a plant to disease (Agrios, 2005).

Pathogenicity genes and disease specific genes are essential for a pathogen to infect a plant (Mansfield *et al.,* 2012). These genes include those which: allow a pathogen to recognise its host; attachment of a pathogen to the surface of a plant; production of infection structures on the surface; invasion of the host (suppression of immune systems); production of toxins, and the capacity to reconfigure plant metabolism for nutrition and reproduction. Therefore, these virulence genes make a microorganism capable of causing disease and when their function is impaired, there is a loss or reduction in symptoms (Agrios, 2005; Boehm *et al*., 2014).

Plant microbe interactions

When a pathogen successfully infects a crop, for example *Phytophthora infestans* commonly known as potato late blight, it can lead to total crop loss and famine (Irish potato famine 1845-1848). We need detailed knowledge on systemic and localised defence to provide agricultural opportunities to increase crop yield by developing pathogen and drought resistant plants, minimising losses from seed to consumption (*Plant cel*l, 2011).

Plants and pathogens have coevolved resulting in complex layers of plant defence mechanisms. Plants have elaborate signalling networks to defend against pathogens (Durrant *et al*., 2004; Pozo, Van and Pieterse, 2004). In turn plant pathogens have developed innovative strategies to modify plant signalling networks by invoking an array of counter tactics. These include hijacking,

Diagram and description from Kazan 2014, figure 1

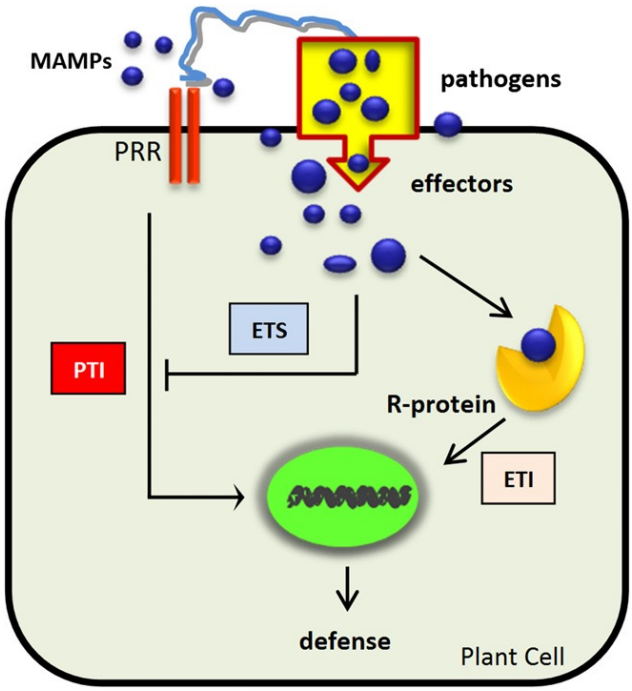


Figure 1. 1, A Simplified view of Plant-Pathogen Interactions

"Pathogen-derived conserved molecules known as MAMPs are detected by plasma membrane–located PRRs, and this recognition triggers PTI. Pathogens interfere with immune signalling through effectors to induce susceptibility, and this is known as effector-triggered susceptibility (ETS). In return, plants have evolved effector recognition proteins (R proteins) that trigger an immune reaction following effector recognition to stop pathogen growth, and this phenomenon is known as ETI."

evading, disrupting hormone signalling pathways and/or crosstalk which is achieved through pathogen-derived hormones known as effectors. These effectors (virulence factors) target plant receptors, transcriptional activators/repressors along with other components to enhance microbial fitness (Vidaver and Lambrecht, 2004).

Plants have an innate immune system whereby transmembrane **p**attern **r**ecognition **r**eceptors (PRRs), such as receptor-like kinases, recognise

microbial- or **p**athogen-**a**ssociated **m**olecular **p**atterns (MAMPS or PAMPS), such as flagellin, invoking **p**athogen **t**riggered **i**mmunity (PTI) (Zipfel *et al*., 2004). After potentially evolutionarily unavoidable PRR detection, the pathogen releases effectors to mask PTI by interfering with PAMP and/or subsequent defence signalling. For example, the **t**ype III **s**ecretion **s**ystem (T3SS) is essential for hemibiotrophic pathogens such as *P. syringae* to deliver effectors. Successful reprogramming of the plants transcriptome, proteome and genome is known as **e**ffector-**t**riggered **s**usceptibility (ETS). See Figure 1.1, 'A Simplified View of Plant-Pathogen Interactions' from Kazan and Lyons, (2014). However, plants have coevolved an arsenal of **r**esistant (*R*) genes encoding intracellular proteins with nucleotide binding domains, but this is not a defining characteristic. Successful R-mediated defence from the plant is known as **e**ffector **t**riggered **i**mmunity (ETI).

Interestingly, strong necrotrophic pathogens which release powerful non-specific toxins and defence suppressing enzymes can also override PTI and ETI processes.

Phytohormones

Plant hormones are critical for defence against biotic and abiotic stressors. Phytohormones are chemical messengers that coordinate cellular activities. The key primary phytohormones include: **ja**smonates (JAs), salicylates (SAs) and ethylene (ET) which are well established. Other phytohormones e.g., abscisic acid (ABA), auxins (indole-3-acetic acid [IAA]), cytokinins (CKs), brassinosteriods (BRs), gibberellins (GA) and strigolactones work alone or in conjunction with primary phytohormones, seen in Figure 1.2 (Kazan and Lyons, 2014; Robert-Seilaniantz *et al*., 2011; Williams, M., 2010; Torres-Vera *et al*., 2014). Some pathogens also produce hormones, but plant derived phytohormones are often synthesised through different biochemical pathways which suggest they evolved independently from one another (Robert-Seilaniantz *et al*., 2011; Kazan and Lyons, 2014). This study focuses on components of the jasmonate and ABA signalling pathways that our laboratory has shown to play a key role in suppressing plant defence (de Torres et al. 2007, deTorres *et al*., 2015, Lewis *et al*., 2015).

Pathogen Secreted Effectors and Toxins

Diagram and text from Kazan and Lyons 2014, Figure 2

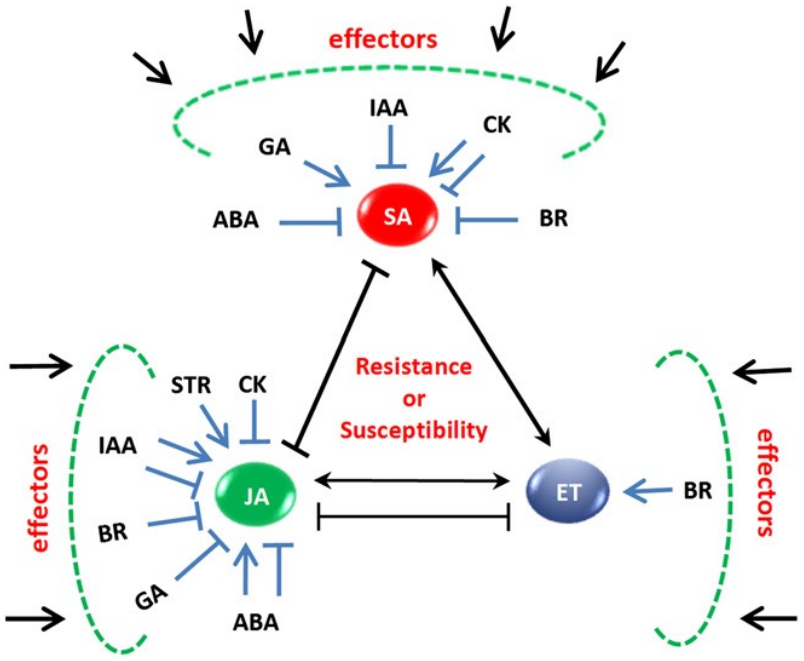


Figure 1.2. Complex Signaling Interactions among Phytohormone Pathways Regulate Both Disease Resistance and Susceptibility in Plants in Attaker-Dependent Manner

The plant hormones Jasmonic Acid (JA), salicylic acid (SA), and ethylene (ET) are primarily involved in plant defence. ABA, auxins (IAA), cytokinin (CK), brassinosteroid (BR), gibberellin (GA), and strigolactones (STR) also regulate plant defence, either alone or in conjunction with the primary defence hormones. Pathogens have developed strategies via their effector repertoire to either interfere with or hijack phytohormone pathways to induce resistance or susceptibility. Forward and blunt arrows indicate positive and negative interactions, respectively.

Pathogen Virulence Strategies

Different host environments are required depending on the type or lifecycle phase of the pathogen i.e. biotrophic or necrotrophic. Necrotrophic pathogens will change the plant environment to promote cell death so it can gain nourishment from those cells. On the other hand, biotrophic cells will prevent

cell (host) death. This complicates host responses, a classical example being that JA and SA pathways can act antagonistically depending on where the host is responding to a biotroph or necrotrophy (review in Thaler, Humphrey and Whiteman, 2012).

Successful microbial pathogens have developed a number of strategies including the production of plant hormones, phytohormone mimics and effector proteins to overcome plant defence. *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) translocates approximately 28 virulence effector proteins into plant cells via the T3SS (Collmer *et al*., 2000; Greenburg and Vinatzer 2003; Cunnac *et al*., 2009). It is highly virulent on *A. thaliana* due to the virulence factors (effectors) delivered through T3SS as they collectively suppress MAMP-triggered immunity and ETI. The suppression enables DC3000 to multiply in the host (ETS) (Ward *et al*., 2010; Macho & Zipfel., 2015). By contrast, DC3000*hrpA*- is mutated in an important structural component of the pilus and prevents the T3SS from functioning (Roine *et al*., 1997). DC3000*hrpA*- triggers MAMP-triggered immunity and can't secrete effectors. This significantly lowers the virulence to *A. thaliana*.

Effectors are often defined as a "*low molecular weight and cysteine-rich protein secreted by pathogens during their interaction with plants and thus are both proteinaceous and non-proteinaceous (e.g. toxins and nucleic acids)*" (Kazan and Lyons, 2014). DC3000 can also produce and secrete phytotoxins (Bender *et al.*, 1999). Phytotoxins are not always required for pathogenicity, but they do enhance pathogen virulence in host plants (Bender *et al.*, 1999). For example, DC3000 produces coronatine (COR), which is a polyketide toxin. This activates the JA signalling pathway through mimicry of jasmonoyl-L-isoleucine (JA-Ile) (Verhage, van Wees, and Pieterse, 2004). COR is required for full virulence in *A. thaliana* and tomato plants (Brooks *et al.*, 2004; Ma *et al.*, 1991; Mittal and Davis, 1995; Zhao *et al.*, 2003).

A common consequence of effector proteins activity is to manipulate the plant phytohormone pathways, as previously mentioned (Schenk *et al*., 2000; Grant reviews 2009 and 2011). By altering such pathways, the pathogen can alter the host's developmental and/or physiological features including stomatal opening

which would allow further infection opportunities (Kazan, 2014). In addition, pathogens cunningly take advantage of intricate crosstalk between phytohormones (Figure 1.2). In this way the pathogen not only changes one phytohormone but also impacts on an array of phytohormones; which can alter both resistance and susceptibility. The pathogen can suppress one hormone to promote another or *vice versa*, which ultimately leads to plant susceptibility.

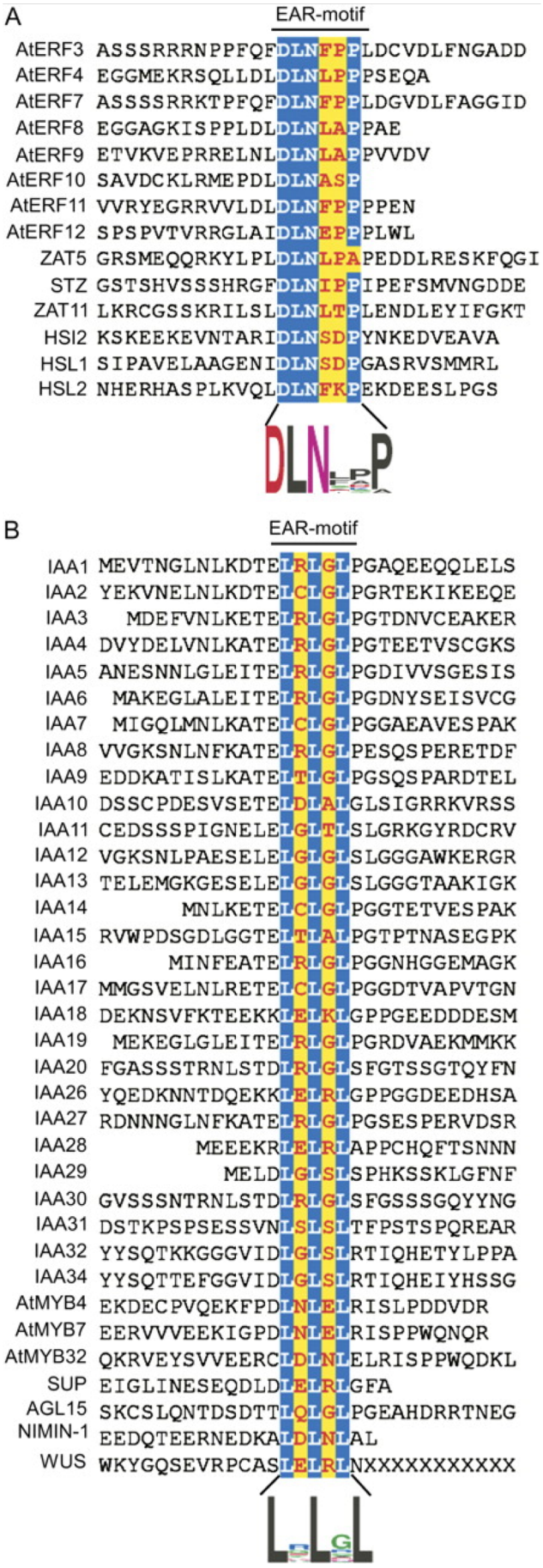
Transcription Factors

Although research into transcription factors is growing, the function and target gene(s) of most transcription factors remain to be characterised (Pruneda-Paz *et al.*, 2014). Understanding such responses is required to improve agricultural yields (Joshi *et al.,* 2016). Transcriptional repressors can contain several domains, for example, DNA-binding domains and repressor domains. Repressor domains, such as **E**RF-associated **a**mphiphilic **r**epression (EAR) motif – the focus of this thesis - is conserved across many plant species, see Figure 1.3 from Kagale and Rozwodowsli (2010). However, its function and cellular fate(s) is currently not well understood, particularly in plant disease and defence. It is likely that the EAR motif has potential novel roles in plant-pathogen interaction and processes other than just transcriptional repression.

The regulation of gene expression is crucial for ensuring developmental programmes and response to environmental stress. Such response includes energy management, organisational maintenance, generating phenotypic variance and response to environmental stress.

Regulatory proteins known as **t**ranscription **f**actors (TFs) underpin the first steps of gene expression. TFs individually or collectively instruct which genes to transcribe, how much, when and where. TFs can be activators or repressors and are characterised by their domains and interaction with other TF proteins, known as co-regulation. TF can not only control other TFs but it can alter expression of itself by positive or negative feedback. Furthermore, a TF may act as an activator or repressor depending on the cellular environment (Ikeda *et al.,* 2009).

Diagram and description from Kagale and Rozwadowski (2010), Figure 1



"Arabidopsis EAR motif-containing proteins described in the literature. The 49 proteins are divided into two groups based on the sequence conservation pattern within the core EAR motif sites (highlighted in color). The alignment includes 12 amino acid residues upstream and downstream of the EAR motif, or up to where the nominal 12-amino acid sequence is abridged by encountering the first or last amino acid of the protein. A, The DLNxxP motif is conserved in some members of class II ERFs, TFIIIA-type ZFPs, and ABI3/VP1 family proteins. B, The LxLxL motif is conserved in AUX/IAAs and some members of the MYB and HD-Zip family proteins. Sequence logos (Crooks *et al*., 2004) illustrating the frequency of amino acids within the EAR motifs are presented below the respective alignments."

Figure 1.3 , A. thaliana EAR motif

Kagale and Rozwadowski (2010) identified DLNxxP motif and LxLxL motif containing transcriptional regulators in *A. thaliana*.

TFs can interact with a specific cis-regulatory DNA sequence and other proteins forming a transcriptional complex, see Figure 1.4 (Alberts *et al*., 2007). Distinct cis-regulatory modules provide the temporal and/or spatial component for the entire gene regulatory region. However, it is important to note that multiple cis-regulatory modules on the same regulatory region are more likely to act together rather than as a single universal promoter element (Benfey and Chua, 1990; Benfey *et al*., 1990; Davuluri *et al*., 2003). When mutating TFs with multiple cis- regulatory regions it is important to construct individual and collective combinations. It has been estimated that up to ten percent of plant genes encode TFs, which is twice the amount relative to animal genomes (Kaul *et al*., 2000; Riechmann *et al.*, 2000; Mitsuda and Ohme-Takagi., 2009; Pruneda-Paz *et al*., 2014). This evidence suggests that TFs in plants have a greater potential to regulate and fine tune gene expression.

Diagrams from The Cell, Fourth Edition, Figure 7.29 (2006)

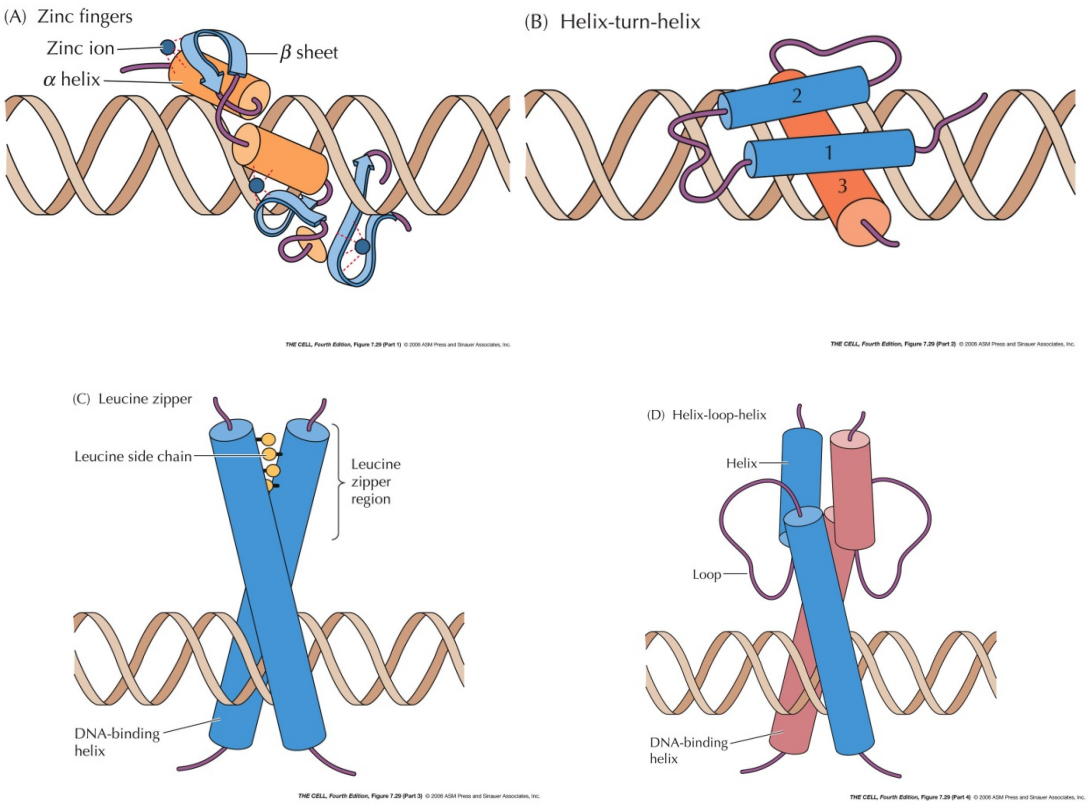


Figure 1.4 , Schematic diagram of Regulation of Transcription factor

There is a gap of knowledge between TFs structural properties and their function (Hiratsu *et al*., 2003). Indeed, determining which TFs are capable of performing protein-protein and protein DNA interactions in *in vitro* studies is one of the major challenges. Furthermore, *in vivo* functional interactions that regulate genes through post transcriptional regulation and TF specificity adds to this complexity. TFs specificity varies; some recognise a broad spectrum of DNA sequences, yet others are more specific. TFs conserved motifs can denote their TF families that are more normally related to function. For example, JAZ proteins have the highly conserved Jas motif that mediates JAZ degradation. Nonetheless, different TF family motifs may overlap making experimental interpretation challenging and this is an area of intense research.

Transcriptional Repressors

A key pathogen virulence strategy is to alter host transcription to promote disease (Lewis *et al*., 2015). TFs can act as switches (on and off) in regulatory cascades (Smith *et al*., 2010) and the ability of pathogens to manipulate or hijack these provides a mechanism to suppress host immunity. One powerful mechanism to rapidly alter a transcriptional immune response would be to remove a transcriptional repressor. One important transcriptional repressor motif which has been identified is the **E**RF-associated **A**mphiphilic **R**epression (EAR) domains (Kagale & Rozwadowski, 2010).

Kagale and Rozwadowski acknowledged "*in recent years, transcriptional repressors have emerged as important elements essential for establishing intricate spatial-temporal patterns of gene expression during plant development and plant responses to stress and hormonal signals*". Transcriptional repressors can contain numerous domains, for example, DNA-binding domains and repressor domains. Repressor domains include the EAR motif which is conserved in plants. Other repressor domains were identified in 29 *A. thaliana* TFs which differ from EAR domains (Ikeda and Ohme-Takagi, 2009). As stated, it's function and cellular fate(s) are currently not well understood (Kagale, Links and Rozwadowski, 2010). The EAR motif potentially plays a novel role in plant-pathogen interaction and processes other than transcriptional repression.

Critically, specific deletion or mutation of an EAR motif found in a TF can abolish the repressive function, therefore modifying the overall function of the TF.

This research focuses on EAR domains in plant immunity, exploring the role of two key transcriptional regulators, described in detail below – a novel MYB transcription factor of unknown function and a specific member of the jasmonate repressor family, JAZ5. Until this work, there was no evidence that HUB37 plays a role in plant pathogen interactions.

The ERF-associated amphiphilic repression (EAR) motif

The EAR motif is a plant-specific active **r**epressor **d**omain (RD). It was first identified in tobacco ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 3 (Ohta *et al*., 2000). The EAR domain is present in many TF associated with plant defence and stress functions (Kazan, 2006). The motif comprises of two small conserved patterns, LxLxL and DNLxxP, giving the amphiphilic feature composed of leucine, an acidic amino acid (Hiratsu *et al.,* 2004), see Figure 1.3. The molecular mechanism of transcriptional repression via the EAR motif is yet to be clarified.

Proteins with EAR motif(s) negatively regulate genes involved in developmental, hormonal and stress signalling pathways that have key biological functions. When an EAR-motif associates with a transcriptional activator they function as a dominant repressor (Hiratsu *et al,*. 2003). This could provide an influential biotechnological tool for human and plant gene expression therapies. Kagale, Links and Rozwadowski's (2010) analysis suggests that the EAR motif is the most predominant form of transcriptional repression so far identified in plants.

It has been suggested that chromatin remodelling may be involved with the function because the EAR motif interacts with one of the most important generic transcriptional regulators, TOPLESS (TPL), and mutations in HISTONE ACETYLTRANSFERASE GNAT SUPERFAMILY 1 suppress the tpl-1 phenotype (Long *et al*., 2006; Szemenyei *et al*., 2008; Mitsuda and Ohme-Takagi, 2009), Notably, TPL and its related "Topless Like" transcription factors play an important role in plant immunity.

MYB Transcription Factors

Transcription factor families like MYB are present in both animals and plants, thus inferring a common unicellular ancestor which have expanded and evolved novel functions through evolution. MYB transcription factors are conserved in many eukaryotes implying evolutionary significance (Nagano, 2000). Recent publications of MYB in humans have linked it to several types of cancers (Grotewold, Chappell and Kellogg, 2015; Fry and Inoue, 2018). Plants have an especially high number of MYB TFs that have evolved unique properties

specific to plants (Richman *et al*., 2000; Pireyre *et al*., 2015). There are 160+ members in *A. thaliana* and 220+ in rice. Due to the plant-specific functions it was hypothesised in 1997 that MYBs are important for plant form and metabolic diversity (Martin and Paz-Ares, 1997). MYB proteins can be passive repressors or passive regulators and they can interact homo- and hetero-dimerize as well as interacting with other proteins.

The MYB TF family is divided into subclasses according to the structure of the DNA binding domain. It contains one, two or three repeats (R1, R2 and R3) consisting of approximately 53 amino acid residues giving rise to a helix-turn-helix structure. The N-terminal R2R3 domain makes direct contact to the major groove of DNA and is highly conserved within the whole family (Dubos *et al.,* 2010). The two clear surfaces allow the TF to bind to the DNA and is available for protein-protein interaction at the same time. This can be described as 'solvent-exposed' for the protein binding site (Grotewold, Chappell and Kellogg, 2015). Current opinion suggests MYB and basic helix-loop-helix TFs evolved in parallel and are associated with developmental and metabolic plasticity (Feller *et al*., 2011). The observation that ectopic expression of a MYB-related TF (AtMYBL) that modulates ABA and salt stress response in *A. thaliana*, causes early leaf senescence and suggests that AtMYBL mediated leaf senescence is mediated by ABA (Zhang *et al*., 2010 and 2011).

There is an evolutionary relationship between rice and *A. thaliana* MYB proteins; MYBS3 plays an important role in both rice and barley seed germination by regulating the depletion of carbon reserves (Lu *et al.,* 2002; Rubio-Somoza *et al*., 2006). In addition, MYBS3 is involved in the cold stress tolerance in rice (Su *et al*., 2010). The understanding of MYB TF has important implications in monitoring gene expression in various scientific areas.

Discovery of the MYB At5g47390 (HUB37) in plant defence

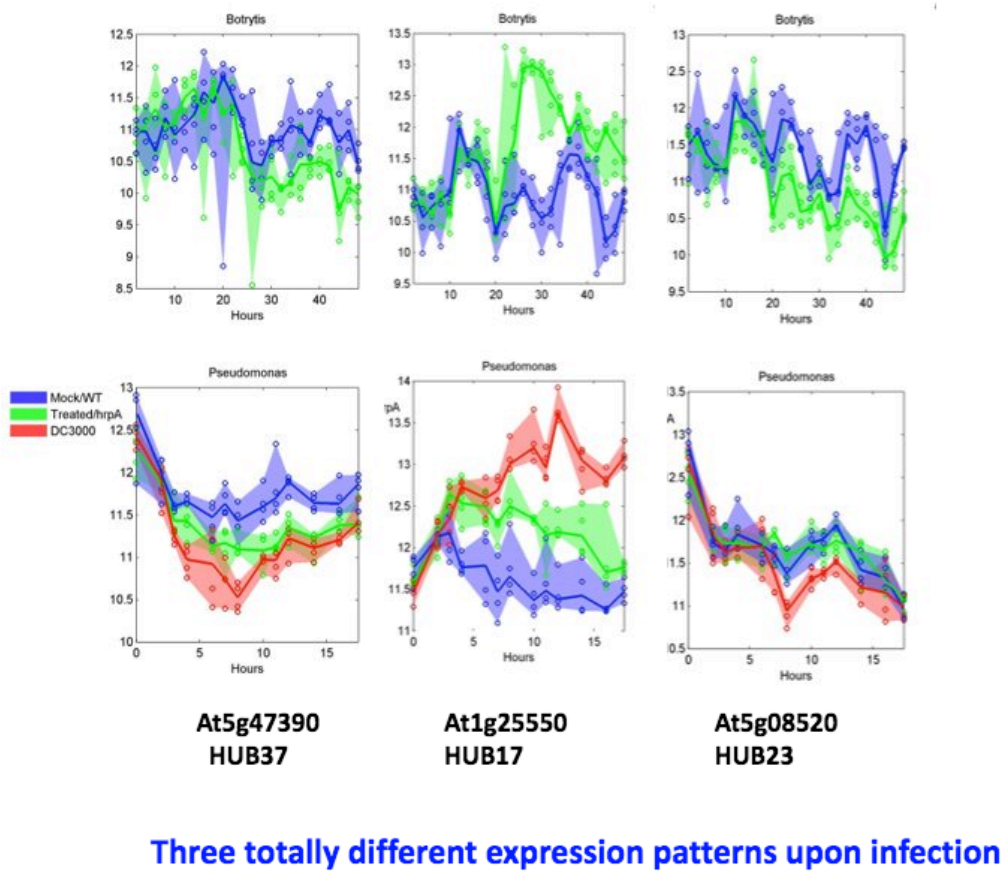


***Figure 1.5.1 , Data derived from the PRESTA consortium* (see Lewis *et al.*, 2015)**

Transcriptomic data of *A. thaliana* gene regulatory network responding to *P. syringae*. Expression pattern of different *A. thaliana* lines over time post inoculation.

Recent high resolution transcriptomic data has provided unprecedented temporal resolution of the *A. thaliana* gene regulatory network responding to the phytopathogen *P. syringae, see Figure 1.5*.1. A microarray gene expression time-course experiments which covered 13 time points over 17.5 h following DC3000 infection identified 1005 TFs differentially expressed suggesting they were targeted by DC3000 effectors. Through detailed network modelling of the data a (then) novel MYB transcription factor At5g47390 (HUB37) was identified to play a significant role in a regulatory network; predicted to be a hub in ABA signalling (sup1. Figure 1). Interestingly, upon further analysis it was found that

MYB TFs closely related to HUB37, HUB17 and HUB23, show significant transcriptional differences in response to DC3000 infection. Notably, HUB23 shows a similar expression to HUB37 but HUB17 has a remarkable contrasting expression patterns.

Lewis *et* al. (2015) reported statistically significant co-expression differences across two of their three conditions; Mock, DC3000*hrpA*- and DC3000 (see Figure 1.5.2). Here they tested for enrichment of TF binding motifs in gene promoter sequences. DC3000 reduced protein accumulation compared to non-virulent DC3000*hrpA*-.

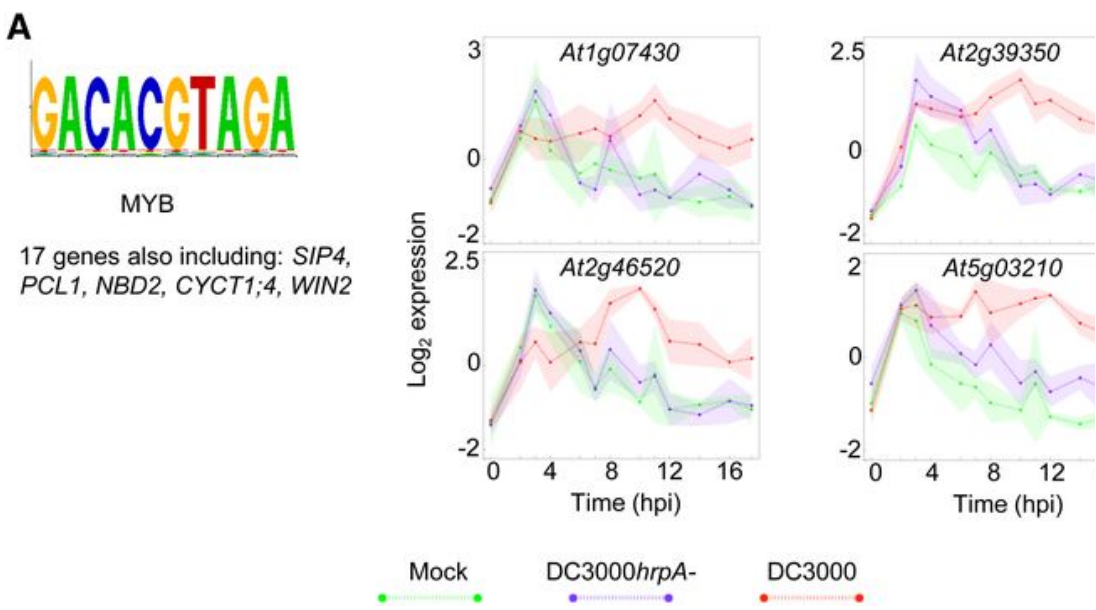


Figure 1.5.2. Time course expression data from Lewis et al., 2015 Figure 7

"Wigwams modules containing genes showing statistically significant coexpression across at least two of the three conditions were tested for enrichment of TF binding motifs in gene promoter sequences. Genes containing enriched motifs in their promoters were identified. In all cases, the mean expression profile of representative genes is shown (green, mock; purple, DC3000*hrpA*-; red, DC3000) with shading indicating SD.
(A) Genes co-expressed during DC3000*hrpA*- and DC3000 infection and containing a MYB TFbinding motif (PLACE: S-000355) in their upstream 500-bp sequences."

HUB37 is predicted to be a highly connected TF in the ABA immunity signalling network

Previous Bayesian State-Space modelling using high resolution microarray time-course data of bacterial infection (Lewis *et al*., 2015) of the ABA perception and signalling network identified HUB37 as a highly connected protein (Figure 1.6).

Grant, unpublished (PRESTA)

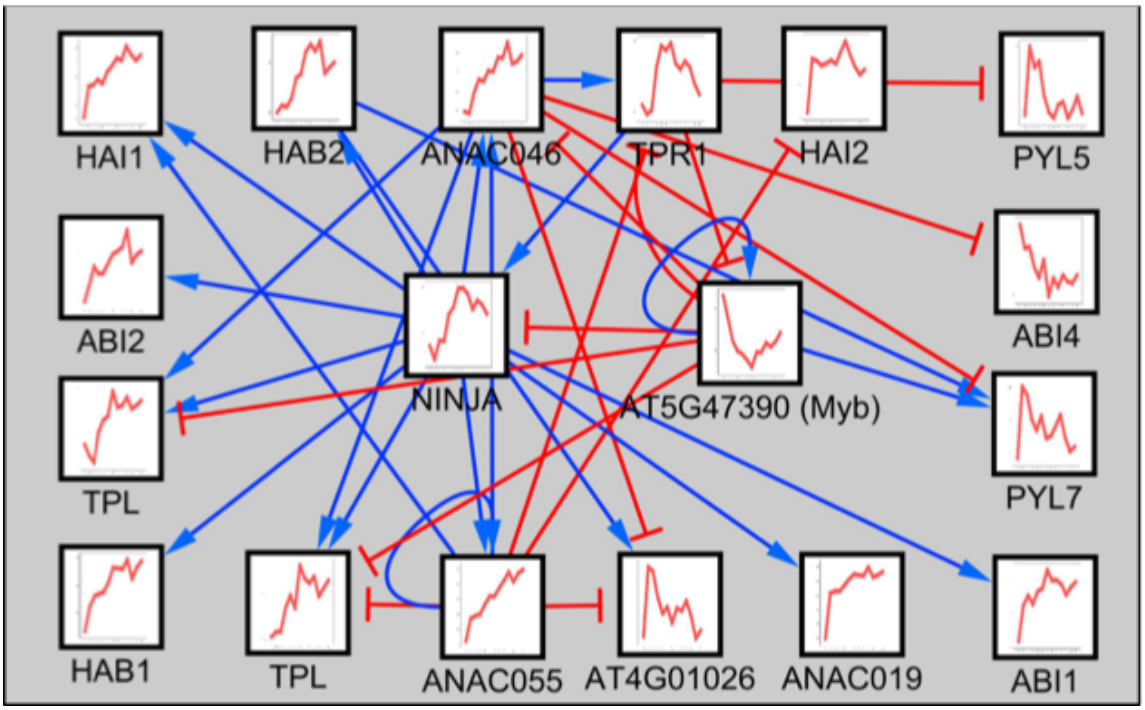


Figure 1.6. Modelling of ABA perception and signalling network with HUB37

AT5G47390, HUB37 appears to be a central node in ABA perception and signalling network.

Interestingly contrary to nearly all the other genes in the network model *HUB37* which encodes an EAR motif, is strongly suppressed during bacterial infection. Within this network HUB37 is predicted to interact with key regulators of ABA signalling and directly or indirectly interact with repressive nodes such as TOPLESS, TOPLESS Related 1 and NINJA (Pauwels, 2010; Kagale and Rozwadowski, 2011). TOPLESS proteins are central regulators of transcriptional complexes and well documented to be involved in mediating plant defence responses (Figure 1.7). NINJA is part of the ABI5 Binding Protein family, originally discovered for its role in ABA signalling but more recently

Diagram and description from Pauwels *et al*., 2010.

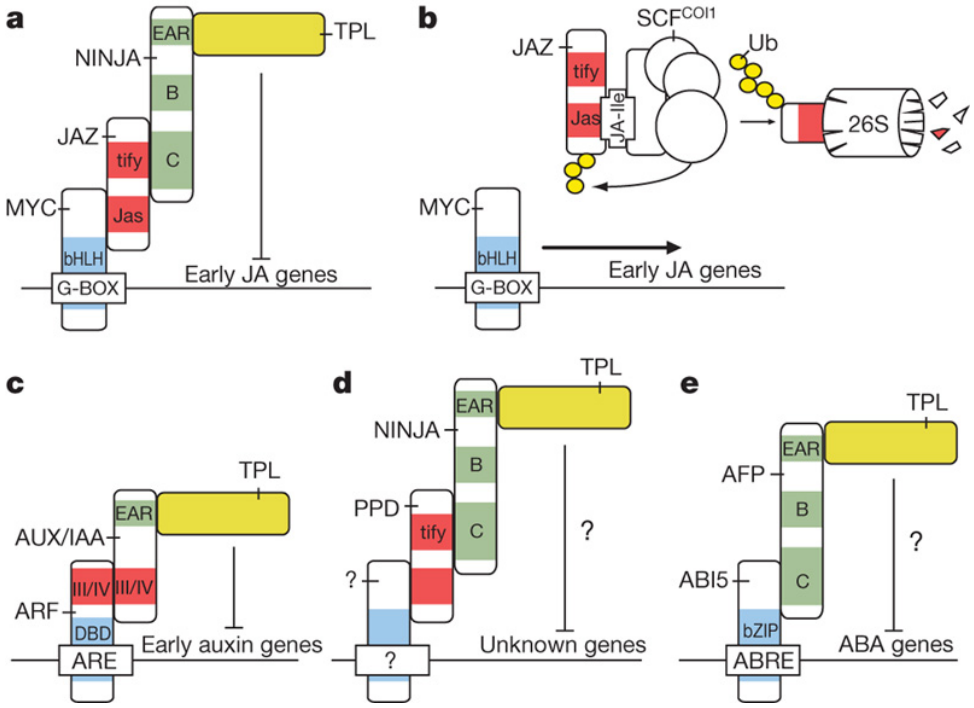


Figure 1.7 , NINJA connects the co-repressor TOPLESS to jasmonate signalling. Model for a general function of TPL proteins in plant hormone signalling

a, In the absence of jasmonates, bHLH MYC factors interact with the Jas domain of JAZ proteins that interact through their TIFY motif with domain C of NINJA. The EAR motif of NINJA is essential for interaction with the TPL co-repressors. b, In the presence of (+)-7-iso-JA-L-Ile, JAZ proteins interact with the ubiquitin ligase SCFCOI1 leading to proteosomal JAZ degradation and subsequent release of the NINJA/TPL complex from the MYC factors and activation of jasmonate-responsive gene expression. c, Jasmonate and auxin pathways are built on similar signalling modules. d, NINJA interacts with other group-II TIFY proteins which might be recruited by yet unknown transcription factors. e, Interaction of the NINJA-related AFP proteins with ABI5 and TPL to regulate ABA responses.

demonstrated to be involved in JA signalling, thus providing a possible mechanism for cross-talk between ABA and JA signalling (Figure 1.7). Thus, from this modelling data it was predicted that the EAR motif could be directly interacting with, and regulating other core genes in ABA signalling, and possibly be involved in JA-ABA crosstalk mediated by co-repressors.

A knockout (GK_783B02, knockout line N65033, stock name: CS365026) of At5g47390 was found to be more resistant to DC3000, see Figure 1.8 [M de Torres unpublished] and this finding was validated (as reported below).

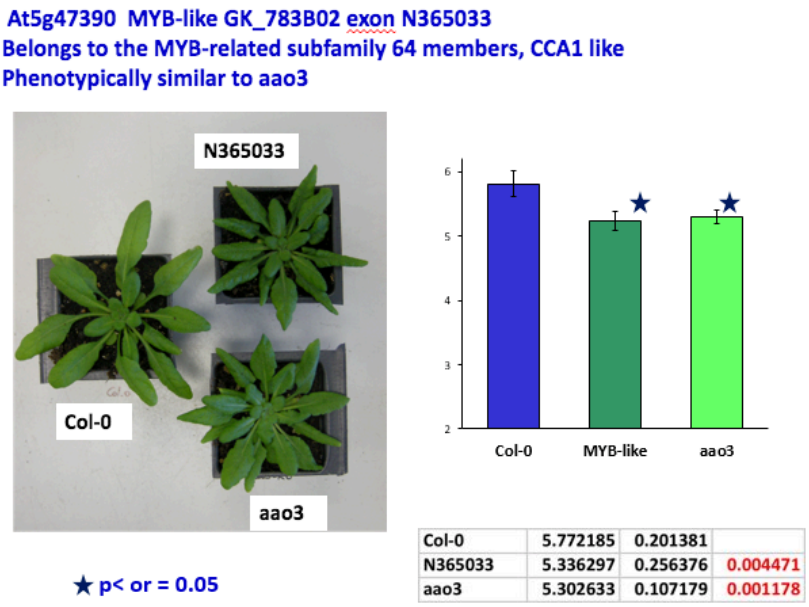


Figure 1.8 , de Torres unpublished preliminary data on At5g47390
A knockout (GK_783B02, knockout line N65033, stock name: CS365026) of At5g47390 was significantly more resistant to DC3000.

Publications in 2013, 2014 and 2015 indicates At5g47390 has a role in a range of diverse plant processes, but to date has not been implicated in plant defence. The first paper, published by Yermin Kwon 2013, named At5g47390 'MYBH'. Over-expression of MYBH caused hypocotyl elongation by enhancing auxin accumulation. This suggested that *MYBH* is involved in the positive regulation of dark-induced hypocotyl elongation. *mybh*, a T-DNA insertion knockout mutant (GK-783B02: NASC ID N365026) had no major phenotypic difference to the wild type Col-0, though we see slightly reduced growth (Figure 1.8). The

mutant, overexpressing line, *MYBH,* increased phytochrome-interacting factor accumulation and thus auxin biosynthesis. *MYBH* phenotypically had darker, curled leaves and increased secondary root number compared to Col-0. MYBH was shown to localise in the nucleus through GFP tagging (Kwon *et al*., 2013). This is consistent with MYB-like transcription factors. At5g47390 contains a motif R/KLFGV. Gibberellin biosynthesis inhibitor, paclobutrazol, blocked overexpression of At5g47390 and increased hypocotyl elongation. In summary, Kwon showed the *MYBH* promoter activates in the dark and *MYBH* transcripts accumulate in the dark.

In a 2014 publication, Dandan Lu identified At5g47390 as 'KUODA1', showing that it was involved in cell expansion, leaf development and final organ size by controlling the expression of peroxidases. The paper reported that At5g47390 is involved in circadian regulation and directly represses genes encoding for peroxidases that control reactive oxygen species homeostasis in the apoplast.

Jasmonic Acid Signalling

The plant immune system relies on complex hormone signalling networks. **J**asmonic **a**cid (JA) is required to adapt to biotic and abiotic stressors. JA is an oxylipin synthesised from the polyunsaturated fatty acid – linolenic acid. It modulates many physiological and developmental agricultural traits such as root growth, survival and fertility (Wasternack and Hause, 2013). It is involved in pollen maturation, growth inhibition and wound induced defence against biotic attacks (Park, 2002; Robson *et al*., 2010). JA inhibits growth processes and is active in reproductive development, pathogen resistance and senescence. JA is an important signal in wounding and pathogen attack which increases both at the site of infection and systemically. JA and **s**alicylic **a**cid (SA) play a central role in defence and have an antagonistic relationship (Glazebrook, 2005; Grant and Lamb, 2006; Gimenez-Ibanez and Solano, 2013). They orchestrate complex transcriptional reprogramming depending on the microbe attack. Necrotophs are more sensitive to JA-defence and biotrophs are more sensitive to SA-defence. JA represses growth and promotes pollen maturation. It is well

established that the JA-dependent defence is crucial in host resistance to *Botrytis cinerea* (Penninckx *et al*., 1996; Thomma *et al*., 1998).

JA and SA mutually antagonise each other via phytohormone crosstalk (Robert-Seilaniantz, Grant and Jones, 2011). The SA pathway confers resistance to biotrophic pathogens. However, activation of this (SA) pathway supresses JA signalling thereby compromising resistance to necrotrophic pathogens. On the other hand, activation of the JA pathway enhances resistance to some necrotrophic pathogens (*Botrytis cinerea*) but inhibits SA pathway and resistance to biotrophic pathogens (reviewed in Thaler *et al*., 2012).

Notably, many strains of *P. syringae* produce the phytotoxin coronatine which is actually a mimic of the bioactive jasmonate JA-isoleucine which binds to the COI1 receptor and targets JAZ proteins for degradation (Geng *et al*., 2014). Coronatine can hijack JA signalling to suppress plant immunity (Robert-Seilaniantz, Grant and Jones, 2011). It is remarkable that a pathogen has evolved a novel small molecule to modulate a key plant hormone signalling pathway.

JAZ proteins directly bind to MYC2 leaving it in a transcriptionally inactive state. In the presence of JA-Ile or COR, JAZs are ubiquitinated by the E3 ubiquitin ligase complex (SCF^{COI1}) and degraded by the 26S proteasome. This causes the release of MYC2 which can then function as a transcriptional activator of JA. This system has a negative feedback loop where by the production of JA represses JA signalling (Lorenzo *et al*., 2004; Melotto *et al.*, 2006; Chini *et al.*, 2007; Thines *et al.*, 2007; Fonseca *et al*., 2009; Zhang *et al.,* 2015). JAZs contribute to early basal and secondary plant defence responses. It has been shown that JAZs can cooperate. For example, JAZ5 and JAZ10 specifically co-operate to restrict COR cytotoxicity and pathogen growth through complex transcriptional reprogramming (de Torres *et al*., 2015; de Torres *et al*., 2016). The *jaz5/10* mutant has a rapid suppression of JA-related components upon bacterial infection compared to other wild-type and other JAZ combinations (Figure 1.9) (de Torres *et al.*, 2015).

Diagram and description from de Torres *et al.*, 2015

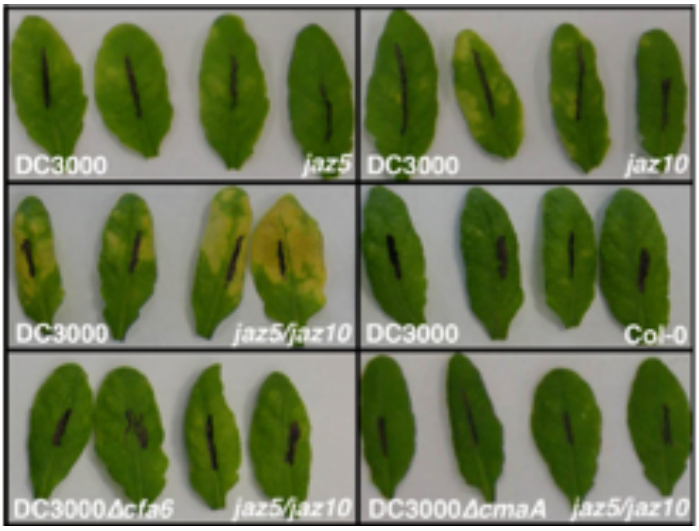


Figure 1.9 , A. thaliana JAZ5 and JAZ10 collaborate to restrict coronatine (COR)-mediated virulence. A jaz5/10 double mutant, but neither single mutant alone, exacerbates the phytotoxic effects of COR.

(a) Infection phenotypes at 5 d post-inoculation (dpi) in (i) jaz5, (ii) jaz10and (iii) jaz5/10 leaves following challenge with *P. syringae* DC3000 (OD600= 0.0005) compared with wild-type Col-0 (iv). Occasional enhanced chlorosis in challenged leaves is illustrated for completeness.

Four out of twelve JAZ members of the JAZ family contain EAR motif(s). JAZ5 contains both LxLxL and DLNxxP in the C-terminal and middle region respectively. The repressor activity of the EAR motif found in JAZ proteins is currently unknown. It is hypothesised that the EAR domain in JAZ proteins is responsible for their dominant repressive function, as found in AUX/IAA.

Contrary to previous understanding, transcription factors are not all activators and when considering transcriptional networks repressors need to be factored into network analyses. The aim of this project was to produce the tools to allow future analysis of the role of repressive EAR domains in plant-pathogen interactions, building on previous research that had implicated two EAR domain

containing proteins as playing a critical role as targets of immune suppression by the bacterial phytopathogen *P. syringae* pv. *tomato* DC3000. It focusses on a novel MYB transcription factor HUB37, predicted to play a central role in the *A. thaliana*-DC3000 transcriptional infection regulatory network. HUB37 contains an EAR domain that has the potential to act as a transcriptional repressor domain. This motif is shared by JAZ5, another transcription factor identified within our laboratory as playing a key node within the jasmonate branch of plant immune suppression, functioning in conjunction with JAZ10 (de Torres *et al*., 2015). JAZ5 contains two EAR domains and we hypothesise it functions differently form HUB37 as JAZ5 does not bind directly to DNA.

Thus two distinct EAR domain containing proteins are, HUB37, that is predicted to have a central role in the immune ABA signalling network and, JAZ5, that functions with JAZ10 to protect the host from pathogen hijacking of jasmonate signalling and are the focus of this investigation. The majority of this project was spent developing tools, transgenic lines with epitope tagged HUB37 and JAZ5 with mutations in the respective EAR domain(s). The HUB37 lines were sufficiently advanced to enable initial characterisation of this MYB TFs role in plant immunity.

MATERIALS AND METHODS

Growth conditions

A. thaliana seeds were sown in F2 compost (Levingthon's, UK*)* and were vernalised for 2 days at 4 ^{0}C in the dark. After vernalisation, seeds were transferred to a short day growth chamber (10 h light, 100-125µEinstein/m^2/sec at 22 ^{o}C day, 20 ^{o}C night). Individual seedlings were pricked after 10 days in P24 celled (5x5 cm) plastic inserts filled with a ratio of 3:1 F2 compost to vermiculite (Willian Sinclair Horticulture Ltd*)* and placed directly into watering trays. Trays were covered with a propagator lid to maintain high humidity for a further 4 days. Plants were grown for 4-5 weeks before experimentation (de Torres Zabala *et al.*, 2003).

Seed Selection

Seed Sterilisation

Seeds were surface sterilised using chlorine gas, by placing in an open microcentrifuge tubes were placed into a desiccator jar with a 250 ml beaker containing 100 ml of bleach in the centre. A total of 3 ml of HCl was added to the bleach and the desiccator immediately sealed and left to stand for 3-16 h (depending on number of samples) in a fume hood. Chlorine gas was released into the fume hood and tubes were left in the fume hood for 5 min to allow evaporation of chlorine gas then left open in laminar hood until the chlorine smell was absent.

Antibiotic Selection

Approximately 100 sterilised seeds were sown on Murashige and Skoog growth media (MS) plates with appropriate selective antibiotic; stratified at 4 ^{0}C for 2 days in the dark and then incubated at 22 ^{0}C ± 1 ^{0}C on a 12 h light 12 h dark cycle (short day) ~100µMol/m^2/s, for 14-16 days (corresponding to a minimum of four true leaves). Individual seedling were then pricked to soil as described above.

Herbicide (BASTA) Selection

Approximately 0.5 ml of seed was evenly distributed across trays (260 mm x 310 mm) filled with F2 Levingtons compost. Seeds were stratified at 4 ^{0}C for 2 days in the dark and then incubated at 22^{0} C ± 1 ^{0}C on a 12 h light 12 h dark cycle (short day) with seed propagator for 10 days. Plants were then treated through root absorption with BASTA

(1:900) [Basta®[1], active constituent 150g/L (13.52% w/w) glufosinate-ammonium]. Plants were observed for the presence of visible phenotypes. Basta-resistant plants were transferred to pots and grown to maturity.

All seed for homozygous selection were taken to at least third generation.

Pathogen Material

Pseudomonas syringae pv. tomato DC3000 (Pst DC3000) strain containing the empty plasmid pVSP61 was maintained on solidified King's B (KB; King *et al.*, 1954) media with antibiotic selection, rifampicin 50µg ml^{-1} and kanamycin 25 µg ml^{-1}. Culture maintenance, preparation and plant inoculation described by Katagira and de Torres-Zabala (Katagiri *et al.*, 2002; de Torres-Zabala *et al.*, 2006; de Torres, Sanchez, Fernandez_Delmond, and Grant 2003).

In planta Pst DC3000 inoculation

Pst DC3000 bacteria were grown overnight in 10 ml liquid KB (King *et al.,* 1954) media containing selective antibiotic at 28 ^{0}C. Overnight cultures were washed and re-suspended in 10 mM $MgCl_2$ to OD_{600} 0.2 (~1 x 10^8 CFU ml^{-1}). Depending on inoculation strategy, dilution series were performed.

Syringe Injection: For bacterial growth curves the DC3000 cell density was adjusted to OD_{600} 0.0002 (~1 x 10^5 CFU ml^{-1}) (de Torres-Zabala *et al.*, 2007).

Undamaged and fully expanded plant leaves were selected, typically three or four per plant. Each side of the central vascular vein on the abaxial surface was nicked with a razor blade and infiltrated using a 1 ml blunt syringe. Excess bacterial solution on the surface of the leaf was gently removed with a paper towel (Katagiri *et al.*, 2002; de Torres-Zabala, 2003).

This method bypasses the effect of stomata closure on the amount of bacteria entering the apoplast. Physiological differences between mutants do not affect the route of entry. CFU and disease symptoms accurately represent plant defence to DC3000.

Spray inoculation: DC3000 cell density was adjusted to OD_{600} 0.02 (≈1 x 10^7 cfu ml^{-1}) in 10mM MgCl and 0.02 % surfactant (*Silwet*) solution. Plants were well-watered the day before to ensure stomata are open. Rosettes were sprayed with bacterial suspension using a spray bottle then placed into a clear bag for 24 h to maintain high humidity, ensuring maximal open stomata (ensure the plastic does not touch the leaf of any plant) (Katagiri *et al.*, 2002).

Plants were kept under normal growth conditions described above during infection. Ecotype Col-0 was used as the wild type control in all experiments (de Torres Zabala *et al.*, 2006 ; Katagiri *et al.*, 2002).

Population counts and data analysis

Plant leaves were infiltrated (OD_{600} 0.0002; six replicates per genotype) and challenge plants grown for three days. Using a flamed sterile disc borer (number 2, 5mm diameter), one disk from each leaf*, with a total of 3 discs per plant were pooled into a 2ml microcentrifuge, containing 1ml of 10mM $MgCl_2$ and a metallic ~5mm ball. Samples were homogenised using a tissue lyser (Qiagen, West Sussex UK) for 2 min at 25 Hz.

For each sample a 10x serial dilution was performed to 10^{-3}. For each dilution, 6 x 10 µl aliquots were plated on KB media Petri dishes (King *et al.*, 1954) and left to dry under a Bunsen burner flame. Plates were incubated at 28 ºC for approximately two days; until bacterial colonies were visible. Bacterial colonies were counted at an appropriate dilution (25-75 CFU/aliquots) under a light microscope. From the six replicates, the average bacterial count and the standard deviation was plotted. Significant differences were determined by Students t-test.

Statistics methods

All bacterial growth measurements were determined from five independent replicates, each comprising three challenged leaves per plant. Significant growth differences between treatments were determined by the Students t-test ($P < 0.5$), error bars representing the standard deviation (SD) of the mean. Experiments were repeated three times.

Western immunoblotting

Total protein extraction

Plant material was placed in a 2ml microfuge tube and snap frozen in liquid nitrogen, and 350µl of extraction buffer (100mM Tris-HCl, pH 7.5, 150 mM NaCL, 1 mM EDTA, 0.2 % Triton 100-X, 2.5 mM DDT, 1 mM PMSF and protease inhibitor cocktail, Sigma) was added to each leaf sample. Using a single metallic ball (5mm tungsten, Qiagen), samples were ground using a tissue lyser at 25 Hz for 2 minutes. The mixture was centrifuged at 12 000 g (max speed) at 4 °C for 10 min.

Protein quantification

The protein concentration for each sample was determined using Bio-Rad protein assay (Bradford, 1976) and samples were equalised with the addition of extraction buffer.

SDS Page electrophoresis

Laemmli sample buffer (5×) was added and the samples were heated for 5 min at 95 ^{0}C. Samples were loaded and separated on 12 or 15% polyacrylamide gels depending on protein size (JAZ or MYB proteins). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 4 ^{0}C.

Western blotting and probing

The blotted membranes were blocked with 5 % semi-skimmed milk powder for 1 h at room temperature (RT) (18 ^{0}C) and probed with the following antibodies: anti-MYC rabbit (AbCam) and anti-HA RAT monoclonal antibody (3F10; Roche) both were used at 1:5000 and 1:10 000 dilutions, respectively, in TBST (Tris/HCl, pH 7.5, with 150mM NaCl and 0.1% Tween 20), for 1 h. Secondary horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-rat antibodies (Sigma-Aldrich), respectively, were applied at 1:20 000 for 1 h before developing the blots with X-ray film using an automated developer.

PCR Genotyping of mutants

Plant genomic DNA extraction

One young leaf was cut with sterile scissors and placed into a microcentrifuge tube. The leaf was crushed with a mirocentrifuge pestle in 500 µl of Shorty buffer (0.2 M Tris-HCL-pH9, 0.4 M LiCl, 25 mM EDTA, 1 % SDS). To this 500 µl of phenol:chloroform was added and vortexed. Samples were then centrifuged at maximum speed (12 000

g) for 5 min at RT (18°C). To a clean centrifuge tube, 450 µl of the upper (aqueous) phase was pipetted into a centrifuge tube containing 450 µl of isopropanol and mixed by inversion to precipitate the DNA. Samples were centrifuged for 10 min at max speed (12 000 g) and supernatant decanted. Precipitated DNA pellets were washed with 200 µl of 70% ethanol, briefly vortexed, decanted and residue liquid removed after a quick spin using a pipette. The DNA pellet was re-suspended in 100 µl of sterile MQ water. The DNA was run on a low EEO agarose gel for quality control and then quantified to determine the DNA concentration.

PCR Reactions

PCR was performed with Taq Polymerase as follows: (Initial denaturation: 95 ^{O}C for 3 min, 35 cycles: 95 ^{O}C for 30 sec, annealing X^{i} ^{O}C for 30sec, extension 72 ^{O}C for X^{ii} sec, end cycle and final extension 72 ^{O}C for 10 min).

X^{i} - Dependent on specific primer combination annealing temperature, optimum established via a gradient PCR.

X^{ii} - Dependent on final extension (1 kb/ min).

Gel electrophoresis

Samples were loaded on 0.8-1.2% agarose gel (low EEO, Melford) containing ethidium bromide (0.2 µg/µl). The percentage of gel was determined by expected amplicon size. Gels were run in a tank with 1x TAE (40mM Tris, 2.2mM Na_2EDTA) buffer. Molecular weight marker, 1 kb DNA ladder (*NEB*), was used as a standard. The PCR products were visualised on a UV transilluminator and photographed.

Construct generation

Constructs for *HUB37, JAZ5* and *JAZ10* were designed with epitope tags.

HUB37 was constructed in a traditional manner using Clontech's C1 pCambia 1032 (10549bp). Golden Gate Cloning technique (Figure 2.1) was used for JAZ construction

as this method enables multiple constructs to be generated using a level system, discussed in detail below.

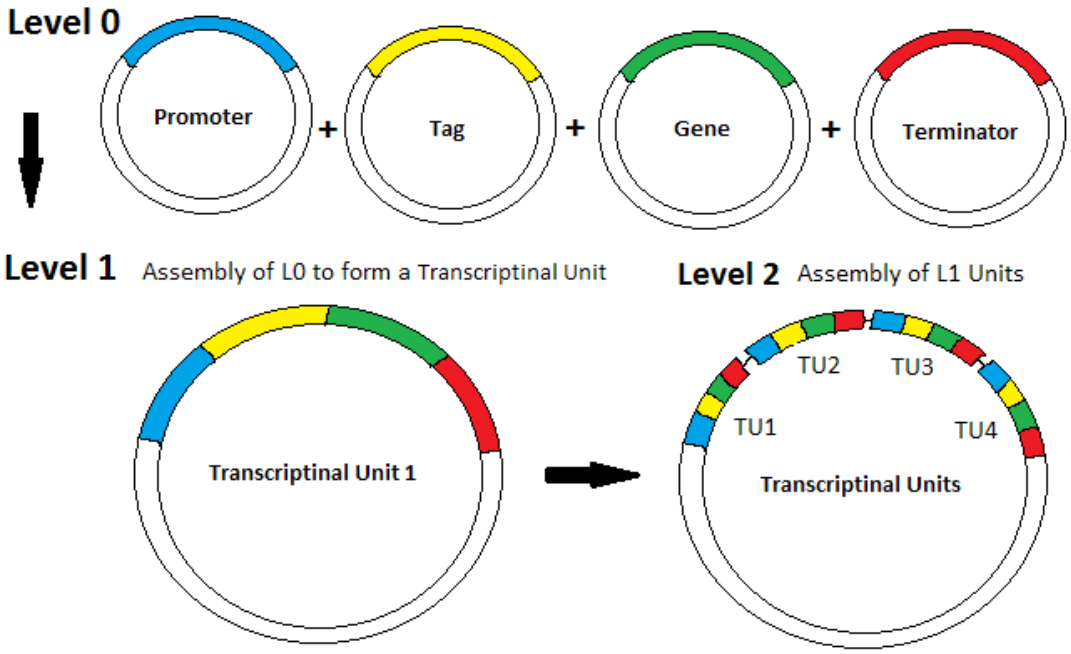


Figure 2.1 Schematic diagram of Golden Gate Assembly

Golden Gate Assembly is based on a modular level system. Using restriction enzymes cutting specific 4bp overhangs; transcriptional units (TU) are assembled using a variety of level zero (L0) vectors with complementary overhangs giving direction. A gene of interest or promoter, for example *JAZ5*, can be inserted into L0 vectors. LO vectors allow a great deal of variation, for example different epitope tags can be swapped in and out. Up to seven TU can be inserted into a level two (L2) vector allowing multiple TU to be inserted into a plant at once. L2 vectors include selectable markers, for example Kanamycin resistance; herbicide resistance cassette can be created in a level one (L1) units.

HUB37 expressed under 35SCaMV* overexpression promoter and was introduced into both WT (Col_0) and *At5g47390* T-DNA insertion KO mutant (*hub37*) for overexpression and complementation studies.

* 35SCaMV promoter drives ectopic expression therefore pathogen phenotypes derived from this line could be hard to extrapolate as the high expression level is likely to distort interactions i.e. unspecific protein-protein/DNA interactions.

A C-terminal hemagglutinin (HA)-tagged was fused to HUB37. This enables identification of HUB37 for *in vivo* Protein-DNA interactions through Chromatin Immunoprecipitation (ChIP)-Seq. The HA tag sequence can be found in supplementary data (Supplementary Data 3).

Cloning strategy for C1-35S$_{pro}$:MYB:3XHA-NOS

Constructs and T0 seed were generated by de Torres Zabala (unpublished). This was cloned into a modified pCAMBIA C1 vector containing a C-terminal MYB.HA tag by vector NcoI (filled) BglII and 5' ScaI and 3' BglII (which removed the stop codon).

1. PCR triple HA from pHB1-HA3 with:
   3HAtag5' +BglII – NcoI: gcta gat ctC ATG GCA GGT TAC CCA TAC GAC (Phusion)
   3HAtag3'+ PmlI and STOP codon, reverse complement: CT TCT CTA CGT TCC TCT tGA caC GTg CAC CGGTGcACGtgTCaAGAGGAACGTAGAG (Phusion)
2. Clone in C1 was cut with BglII + PmlI: C1-3HA
   PCR At5g47390 from cDNA with 5' primer ScaI (AGT ACT blunt) + and 3' primer + BglII (removing STOP codon)
3. Clone in C1-3HA was cut with NcoI filled and BglII

HUB37 ORF (1098bp) was amplified using cDNA with primers introducing EcoRV sites, then cut with EcoRV and cloned in Cambia 1302 (C1) cut with NcoI filled and PmlI.

Primers:

At5g47390-START ggGATATCATGACTCGTCGATGTTCTCACTG;

At5g47390-STOP cctcagAtAtCTTATAAAGCGTGTATCACG.

Yeast two-hybrid Assay (Y2H) for protein-protein interaction against a general library of proteins.

Future work envisaged interactors would be confirmed by an in vivo pull down experiment, for example Tap-Tag to see in planta protein-protein interactions.

HUB37 Protein Degradation *in vitro* with DC3000

To test this, initial experiments were conducted that involved non-inoculated total leaf protein extracted from HYB37:HA tagged lines being mixed in a 1:1 ratio with DC3000 inoculated Col-0 leaf (non-transgenic) protein extract. Mixtures were incubated at room temperature (18 ^{o}C) for up to 18 h before SDS loading buffer was added to stop the reaction. It was hypothesised that HUB37 would degrade at a faster rate with Col-0 inoculated total protein extract compared to non-inoculated total protein extract due to the defence response. This was the first attempt to test if the abundance of HUB37 decreased over time *in vitro* as previously found *in planta*.

Golden Gate Assembly of *JAZ 5, JAZ10* and *JAZ5* EAR mutagenesis.

Golden Gate Assembly multiple constructs were constructed and transformed into plants using agrobacterium GV3101 as described by Holster, 1978. Molecular assembly strategy followed as described by Engler and Weber (Engler, Youles and Gruetzner, 2008; Engler *et al.,* 2014; Weber *et al*., 2011). Please refer to Golden Gate Cloning strategy diagram for level assembly strategy (Figure 2.1).

JAZs contribute to early basal and subsequent secondary plant defence responses (de Torres, 2015). Detailed genetic analyses revealed that *JAZ5* and *JAZ10* function co-operatively compared to other JAZ proteins (de Torres Zabala, 2015). Their co-operative behaviour attenuates phytotoxicity mediated by the bacterial phytotoxin coronatine (COR) and to moderately restrict bacterial growth (de Torres Zabala, 2015). For this reason, *JAZ5* was proposed to be tested on its own and with *JAZ10* to establish if the JAZ5 ear domains affected the co-operative behaviour.

Different N-terminal epitope tags were used for *JAZ5* and *JAZ10* to localise each protein *in planta*. It has been reported that *JAZ10* has four splice variant proteins (Chung *et al*., 2010) and for this reason an N-terminal tag was used for both *JAZ5* and *JAZ10*). The native promoters of ~1200bp were used to ensure usual hormone

responses and not to impact on the fine-tuned signalling network. This allowed direct comparison to WT and KO responses to the pathogen.

The native *A. thaliana Actin2* terminator sequence (*Act2 ter.*) was used to terminate expression for *JAZ* constructs.

Positive colonies were selected through PCR amplification and digestion before being sequenced (Eurofins Genomics, Tube Sequencing).

JAZ 10

The *JAZ10* promoter was amplified by High-Fidelity PCR (~1500bp), while primers with BpiI recognition overhang sites, and digested and ligated into L0 acceptor vector pAGM1251 with BpiI and T4 DNA ligase.

The *JAZ10* Gene (1502bp) was amplified as above and ligated into pICH41808. 3x MYC (122bp) tag and *Act2* ter. (485bp) were sourced from Golden Gate Modular Cloning Toolbox for Plants, pICSL30009 and pICH44300 respectively.

Following sequence validation, all L0 vectors were amplified in DH5a and linearised. A one-pot digestion and ligation reaction was performed to assemble the contigs into a Level one position 1 vector. After further selection, amplification and sequencing the *JAZ10* cassette (7996bp) was ligated into a Level 2 vector along with a BASTA resistant cassette (11138bp) generating pGBKT7::*JAZ10*$_{pro}$:MYC:*JAZ10*:Act2-BASTA.

The resulting construct was introduced to *Agrobacterium* competent cells (GV3101) using heat shock transformation method as described by Holster, 1978.

JAZ 5

Like JAZ10, the JAZ5 native promoter was amplified by High-fidelity PCR (~1500bp). The *JAZ5* gene (~1400bp) was inserted into L0 acceptor vector pAGM1276, digested with BpiI and ligated in three sections. The BsaI restriction site was removed by Kit based mutagenesis in the L0 vector. Further mutagenesis was used to mutate one or both EAR domains using a series of appropriate primers (Figure 2.2). A different approach using pICH86966 was developed to assemble Level two Position two for *JAZ5* WT, eari and eari/ii. In this approach, L0 constructs were ligated directly into pICH86966 with Kanamycin resistance, avoiding L1 construction. 3x HA (124bp) tag and *Act2* ter. (485bp) were sourced from Golden Gate Modular Cloning Toolbox for Plants, pICSL30008 and pICH44300 respectively.

The resulting constructs were introduced to *Agrobacterium* competent cells (GV3101) using heat shock transformation method as described by Holster, 1978. Col-0, *jaz5, jaz10 and jaz5/jaz10* plants were transformed with *Agrobacterium* containing the relevant construct by floral dipping.

pGBKT7::*JAZ5*$_{pro}$:3xHA:*JAZ5*:Act2 - kanamycin

pGBKT7::*JAZ5*$_{pro}$:3xHA:*JAZ5*eari:Act2 – kanamycin

pGBKT7::BASTA-*JAZ5*$_{pro}$:3xHA:*JAZ5*earii:Act2.

pGBKT7::*JAZ5*$_{pro}$:3xHA:*JAZ5*eari/ii:Act2 - kanamycin

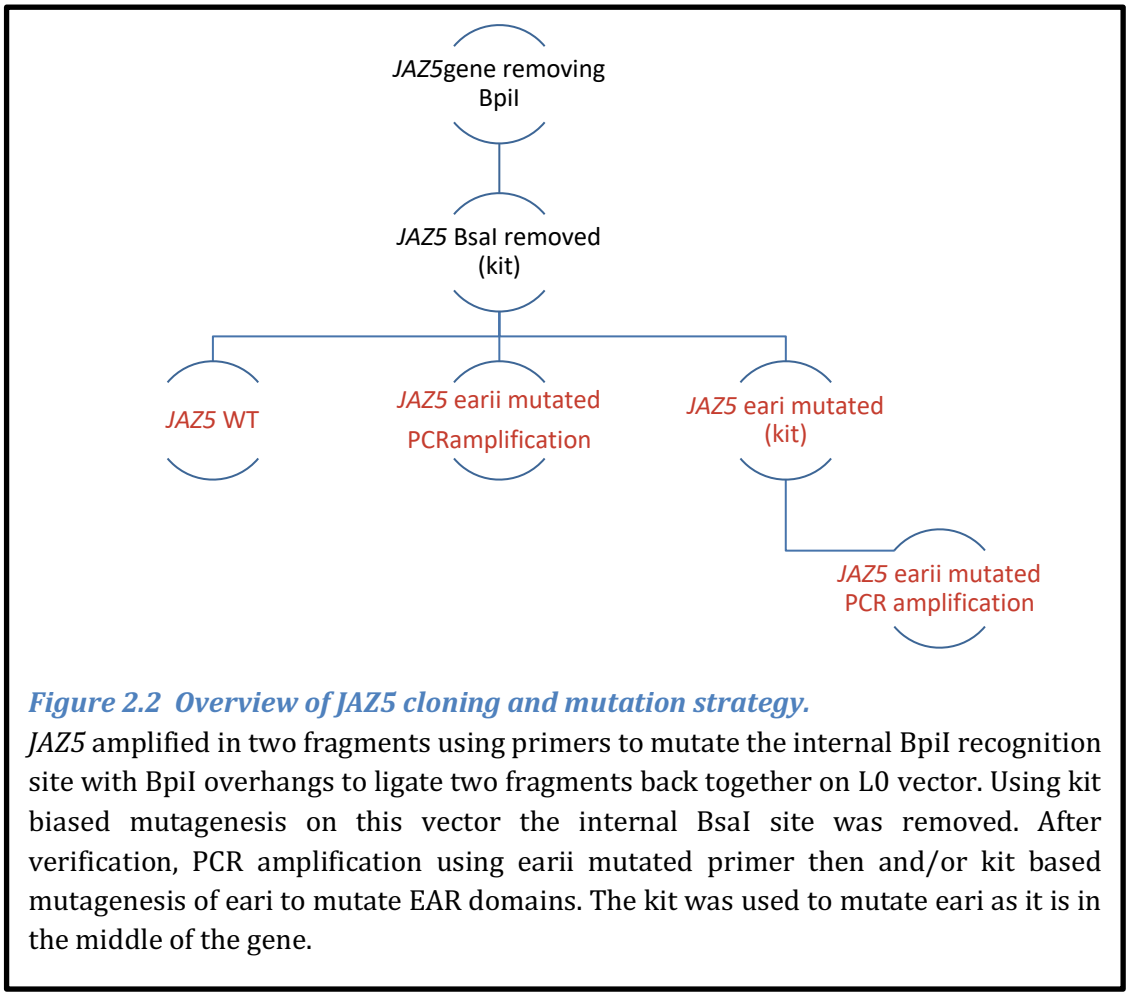


Figure 2.2 Overview of JAZ5 cloning and mutation strategy.
JAZ5 amplified in two fragments using primers to mutate the internal BpiI recognition site with BpiI overhangs to ligate two fragments back together on L0 vector. Using kit biased mutagenesis on this vector the internal BsaI site was removed. After verification, PCR amplification using earii mutated primer then and/or kit based mutagenesis of eari to mutate EAR domains. The kit was used to mutate eari as it is in the middle of the gene.

Plant Transformations

Transformations were performed with *Agrobacterium tumefaciens* strain GV3101 by the floral dip method (Clough and Bent, 1998).

Results

This results section covers the generation and testing of constructs that provide the foundation for understanding the role of EAR domains in plant pathogen interactions. As I initiated this project, the laboratory gained verification via *in planta* bacterial growth assay that HUB37 indeed had a biological role in suppression of plant defence due to increased resistance of a loss of function HUB37 allele following DC3000 challenge.

HUB37 Mutant Phenotype

One initial task was validating a biological role for HUB37 in plant defence. A T-DNA insertion mutant line (GK_783B02, knockout line N65033, stock name: CS365026) in *HUB37* (*At5g47390*) was generated and tested to be homozygous. This line was shown to be significantly more resistant to *P. syringae* DC3000 compared to WT Col_0, Figure 3.1.

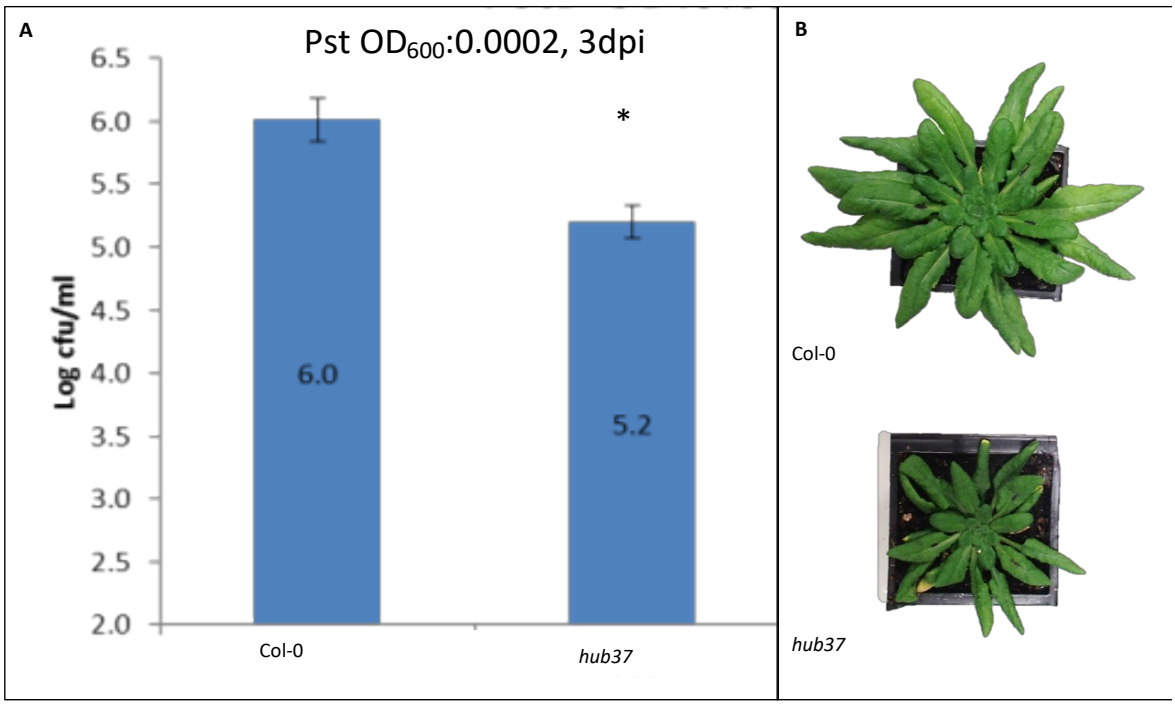


Figure 3.1. HUB37 is significantly more resistant than Col_0

A) Plants were inoculated with DC3000 0.0002 OD_{600} and population counts undertaken 3dpi. Enumerating bacterial counts indicates that *hub37* is significantly more resistant than WT, students t-test ($P < 0.05$). B) *hub37* has smaller leaf size compared to WT.

HUB37 Selection Process

Transcription profiling showed a strong suppression of *HUB37* expression during infection with DC3000. In order to determine how well the abundance of HUB37 protein mimicked the transcript levels during suppression of immunity, plants were transformed with the C1-35S_{pro}:MYB:3XHA-NOS and homozygous lines selected.

The C1-35S_{pro}:MYB:3XHA-NOS construct, was transformed into Col-0 or *hub37* knockout lines and T0 seed generated by de Torres Zabala using standard protocols. Transformants from T_1 seed were selected on hygromycin MS plates, grown in soil, selfed and then the T_2 generation selected for homozygous lines on hygromycin MS plates, looking for 100% germination. Independent lines were then tested for HUB37 accumulation by Western blot of crude protein extracts with an antiHA antibody. Not all T_2 plants expressed MYB:HA, when tested through Western blot and plants positive on Western's were taken to the T_3 generation (see Figure 3.2). Three independent lines for each construct were taken forward for further analysis. Phenotype of each line and levels of expression were selected in lines and a representative example of these are shown in Figure 3.3.

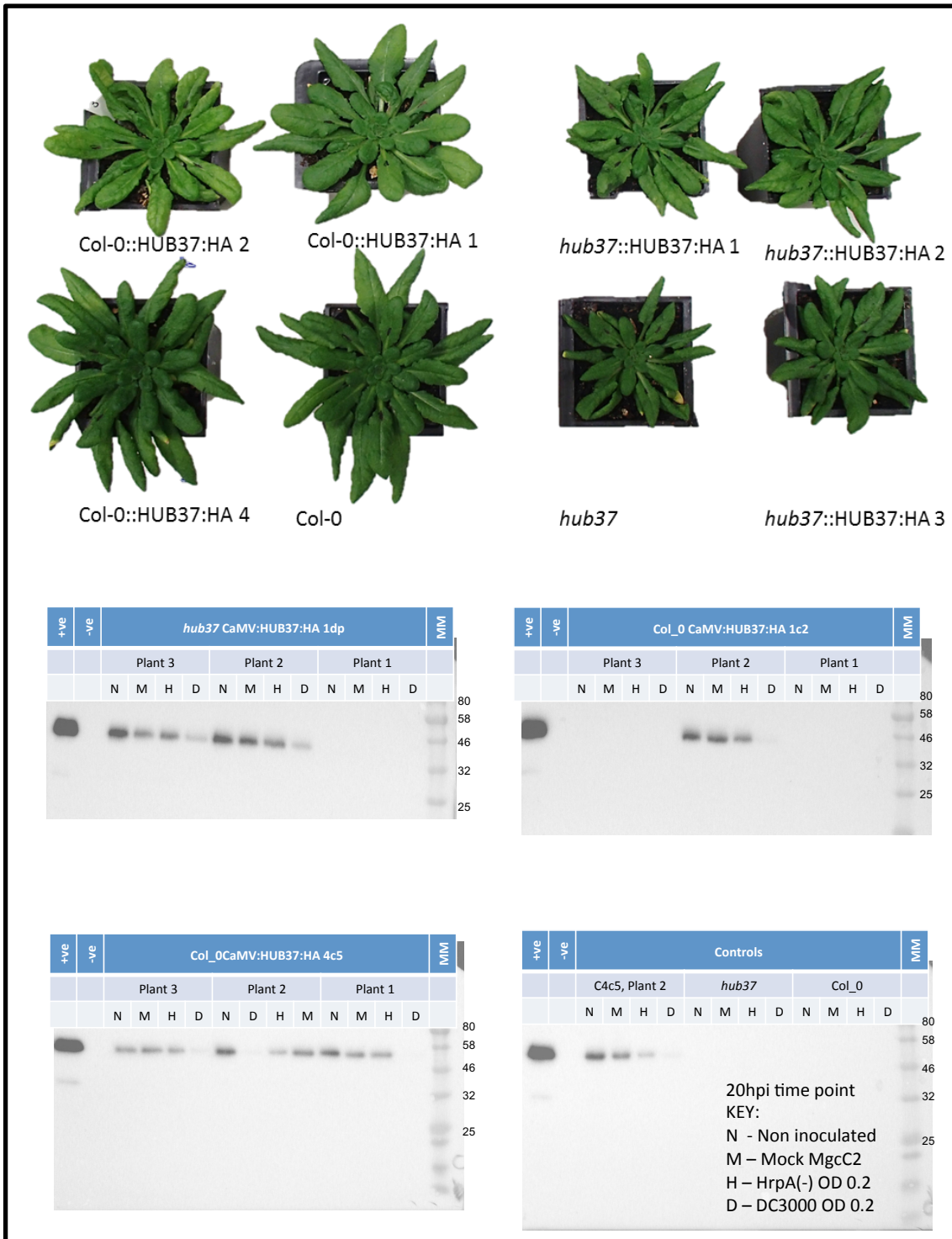


Figure 3.2. Accumulation of HUB37 in first generation (top) and third generation (bottom left, 4c5) transgenic lines.

Col-0 leaf phenotype larger than *hub37.* T_1 generation of transformed *hub37* plants, with overexpression of HUB37:HA, appear to be larger than *hub37* and similar to WT (Col-0). Col-0 with over expression of HUB37:HA do not appear larger, some in fact were smaller. Western Blot analysis, probing for anti-HA, indicated expression in the T_3 generation Col-0::CaMV:HUB37:HA (T_1 4, T_2 C,T_3 5). MYB:HA is degraded in the presence of DC3000 20hpi.

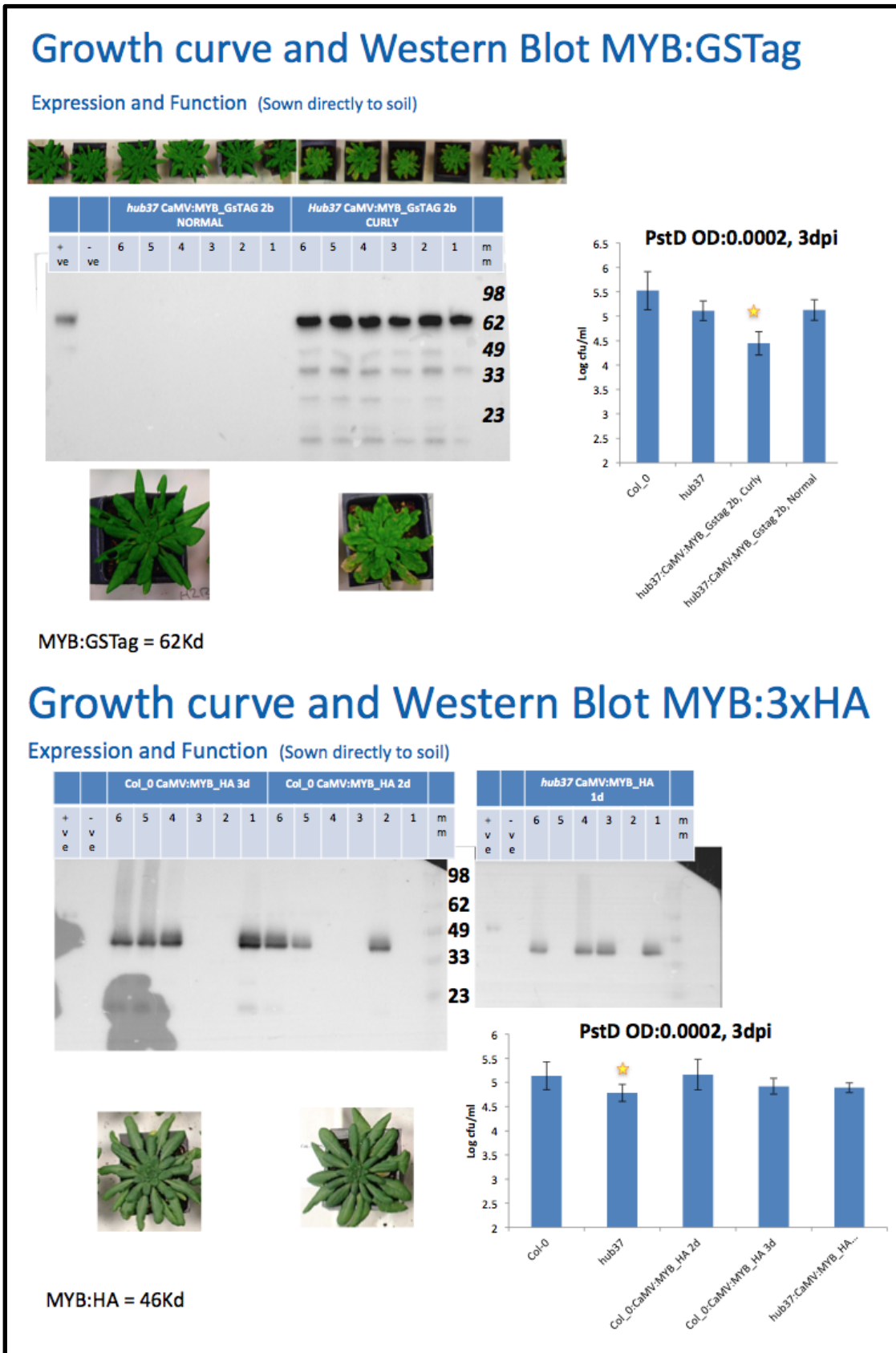


Figure 3.3 T_3 generation phenotype and levels of expression selected in lines

HUB37 Accumulation Decreases Over Time When Plants are inoculated with DC3000

Figure 3.2 suggests that HUB37 protein is not stable following DC3000 infection compared to DC3000hrpA, mock or unchallenged leaves. To investigate this further leaves from selected transgenic HUB37 lines were inoculated with *P. syringae pv. tomato* strain DC3000 (0.2 OD_{600}) and protein extracted over a time-course. Western blot with anti-HA antibody revealed that HUB37 protein appeared to degrade over time. Interestingly, over the time course, as the ~46Kd HUB37 disappears a smaller immunogenic protein band appears (Figure 3.4).

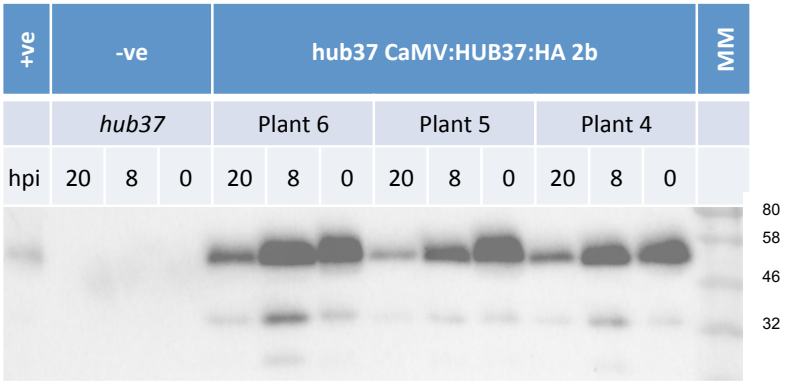


Figure 3.4. HUB37 protein decreases over time when plant tissue is inoculated with DC3000

Western blot analysis showed HUB37 protein degrades (reduces) over time when plant tissue was challenged by DC3000. Leaves were inoculated with DC3000 0.2 OD_{600} and snap frozen at 0, 8 and 20hpi. Proteins were extracted, normalised and run in a 10 % SDS-acrylamide gel. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane before being probed with hemagglutinin (HA) antibody for the HA tag on HUB37. Then a Horseradish peroxidase (HRP) secondary antibody enabled florescent imaging of HUB37 protein expression, depicted by the dark bands.

Virulent DC3000 caused HUB37 protein to diminish compared to non-virulent *HrpA(-)*

Figure 3.5 shows the level of HUB37 accumulation in 3 lines of one independent transformant (note plant 3 appears not to be transgenic). In line with previous microarray gene expression time-course data DC3000 reduced HUB37 accumulation compared to non-virulent DC3000*hrpA* (Figure 1.5.2) *(Lewis et al., 2015)*, indicative of type III mediated degradation of HUB37. To try to quantitate the levels of HUB37, grey-scale scale analysis was used. In grey scale analysis quantifies brightness as a percentage, 0% is black compared to 100% being white; non-inoculated expression on average was 35%, Mock 49% (data had a comparatively lager range of 16%) DC3000*hrpA* 49% and DC3000 75%. Repeat experiments can be found in Supplementary Data 3.3.

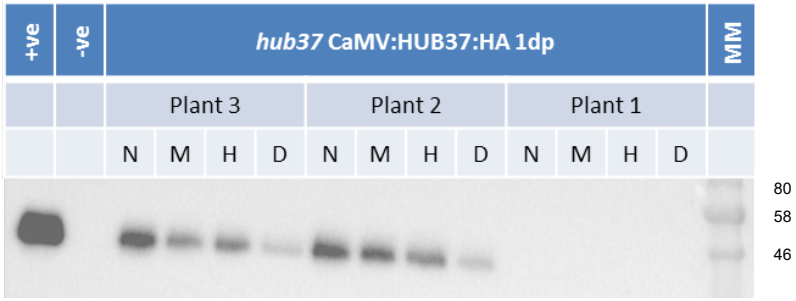


Figure 3.5. HUB37 protein expression when challenged with DC3000 and hrpA
20 hpi time point of HUB37 abundance when challenged with DC300, DC3000h*rpA* or mock. Key: N - Non inoculated; M – Mock MgCl2; H – DC3000*hrpA,* OD_{600} 0.2; D – DC3000 0.2 OD_{600}. Using grey scale analysis, N - 35%, M – 49%, H - 49% and D - 75% indicating the brightness of black for each section.

HUB37 Protein Degrades at the same rate *in vitro* with DC3000

Due to the abundance of HUB37 decreasing over time during a DC3000 challenge it was hypothesised that it could be due to specific proteolytic activity activated by type III effectors. Abundance of other transcriptional repressors involved in plant defence, such as JAZ proteins, are controlled through ubiquination by the 26S proteasome (Thines *et al*., 2007). While this was only repeated once, Figure. 3.6 shows that the abundance of HUB37 decreased over time *in vitro* as previously found *in planta*. Expression was not detectable in the Col-0 background lines (Supplementary Data 4.5). Future experiments can focus on optimising the method by altering the extraction buffer, temperature and increasing the concentrations of proteins (for Col-0 background) which may alter the enzymatic activity and provide higher sensitivity to help validate this exciting result.

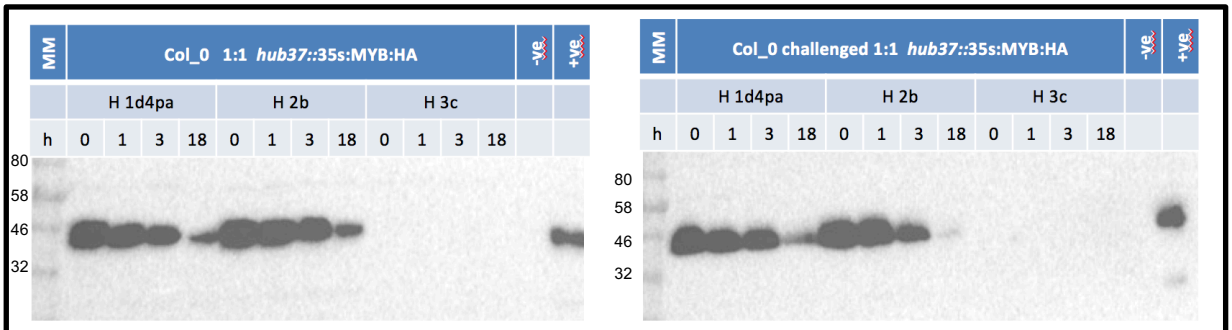


Figure 3.6. in vitro DC3000 inoculation,* HUB37 *Degrades at the same rate

Degradation of HUB37 *in vitro* over time; Col-0 DC3000 inoculated protein extract was mixed in a one to one ratio with non-inoculated HA:HUB37 expressing lines. Clear degradation can be seen in the *hub37* background over time. Challenged protein mix appeared to degrade more by 18 h compared to non-inoculated tissue. Panels show two independent experiments.

JAZ Cloning

Using Golden Gate cloning (see materials and methods section) *JAZ5* and *JAZ10* were expressed under their native promoters and epitope tagged in the N-terminus, as the C-terminus is implicated in key protein-protein interactions, and in JAZ10 alternative spliced C-terminal variants.

These provided the foundation for me to develop lines with JAZ5 EAR domains knocked out in various combinations, see Figure 3.7.

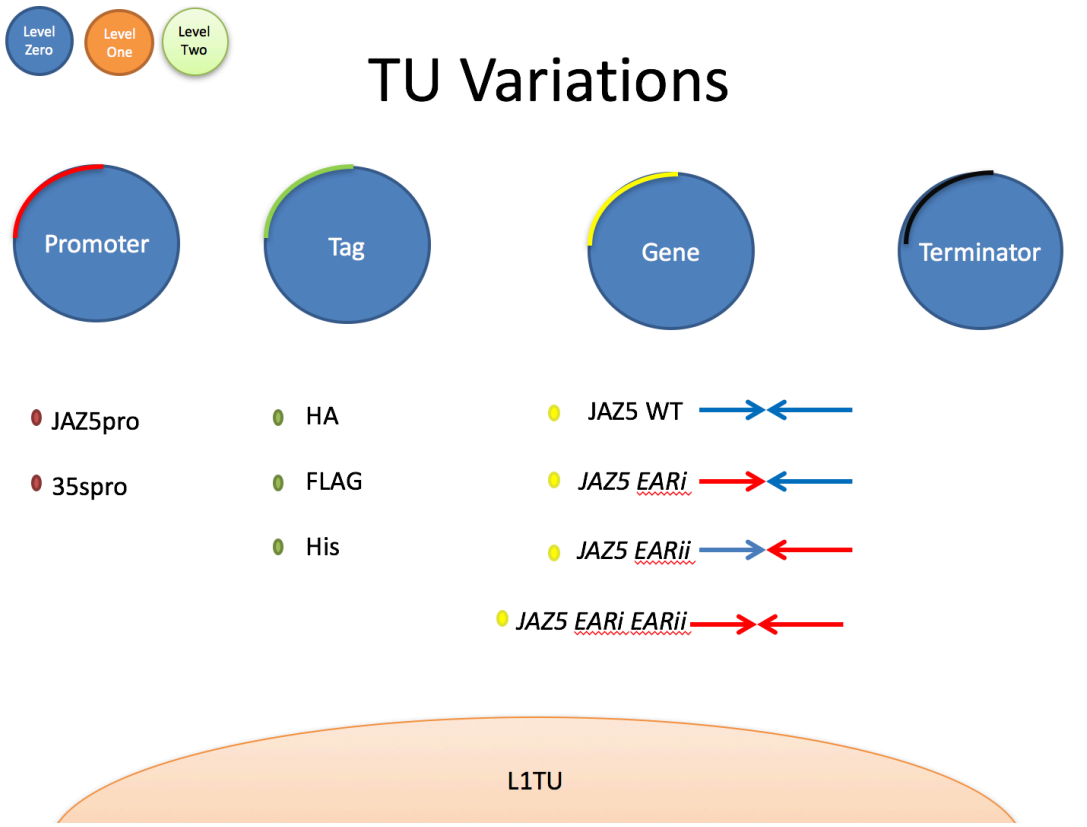


Figure 3.7. Schematic diagram showing transcriptional unit variations for JAZ5 EAR domain mutation combinations.

A combination of primers to amplify and mutate EAR domain i and ii in JAZ5. Golden Gate assembly transcriptional units (TU) could then be assembled with a choice of epitope tag and promoters.

JAZ10

After level zero (L0) vector assembly with the *JAZ10* promoter it was sequenced from left and right vector borders across the whole inserted amplified promoter. This revealed an adenine insertion at ATG -1445 bp, see Figure 3.8. As this insertion was in the promoter with over 1kb distance from the gene, the process was not repeated and the vector was used later for Golden

Gate assembly. No mutations occurred across the full length of *JAZ10* CDS which was sequenced in full (1502bp). Before proceeding to **l**evel two (L2), level 1 *JAZ10* promoter, N terminal 4X MYC tag and *JAZ10* gene junctions were sequenced which confirmed *JAZ10* is in frame with the MYC tag, see Figure 3.9. Confirmation by PCR diagnostics and digestion showed position 1 MYC:*JAZ10* cassette, position 2 BASTA cassette and end link 2 successfully ligated together in pGBKT7. The construct was successfully transformed into *Agrobacterium* competent cells GV3101 which were used for floral-dip transformation into Col-0, *jaz5* and *jaz5/10* plants. T_0 seed was harvested and positive transformants identified through BASTA selection.

Figure 3.8 An insertion in JAZ10 promoter

At ATG-1445 an extra adenine inserted was found though sequencing indicated by the red box compared to the expected aligned sequence.

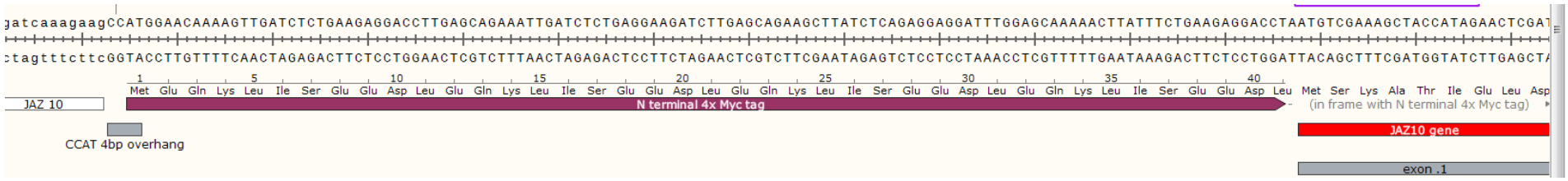


Figure 3.9 N terminal 4xMYC epitope tag in frame with JAZ10 gene.

The *JAZ5* promoter and an N terminal MYC epitope tag and gene successfully ligated together; *JAZ5* gene was in frame with MYC tag.

JAZ5 and EAR mutation

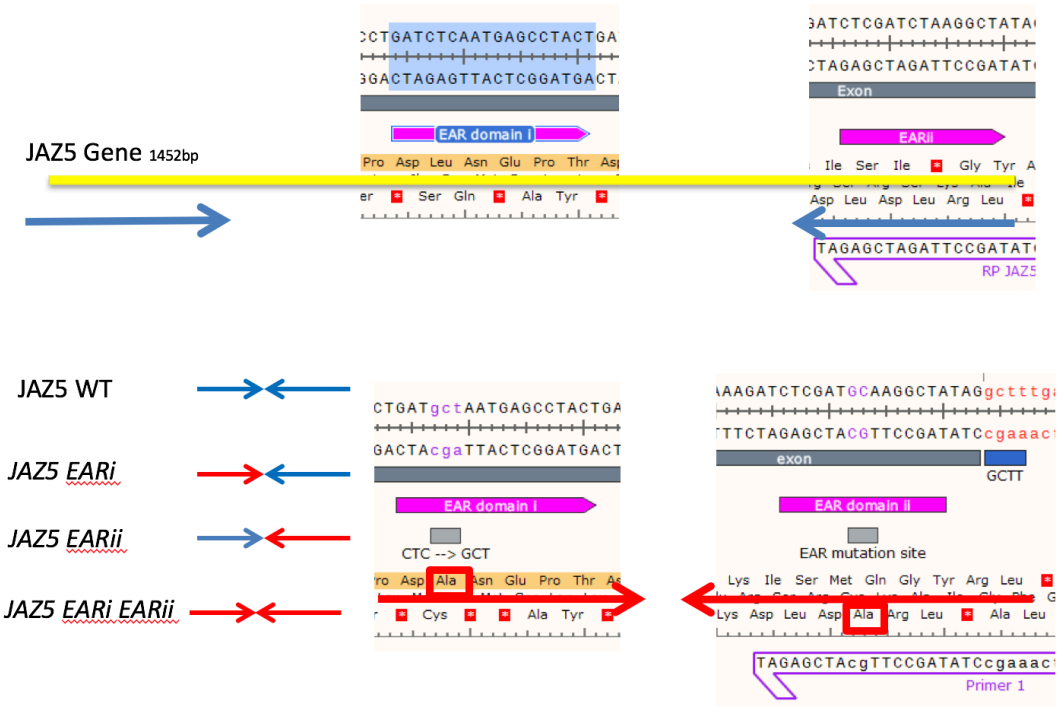


Figure 4.0. Schematic diagram showing primer combination to achieve JAZ5 EAR domain mutation combinations.

Through Golden Gate Assembly and kit-based mutation using Quick-Change II site-directed mutagenesis kit (Stratagene), WT and mutated EAR domain(s) were constructed and expressed in WT (Col-0) and KO *jaz5* and *jaz5/10* double mutant lines (see Figure 4.0). All constructs were sequenced to confirm the corresponding mutation and assembly (see Supplementary Data 5 for primers and Figure 4.1 for amino acid changes).

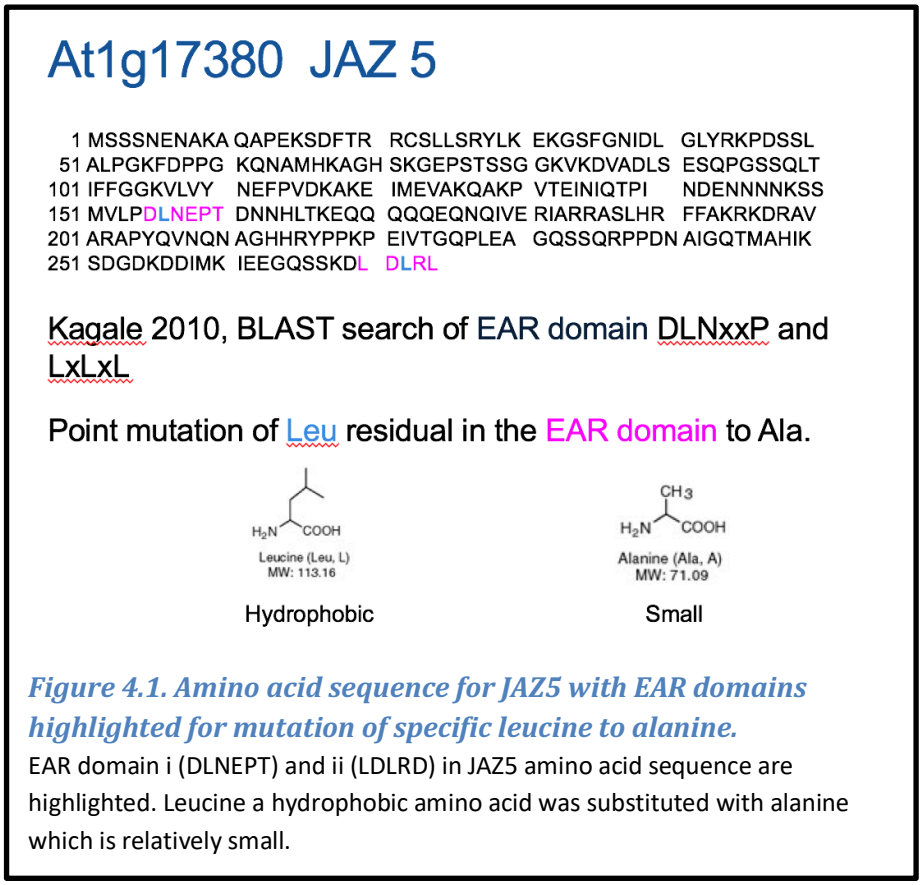


Figure 4.1. Amino acid sequence for JAZ5 with EAR domains highlighted for mutation of specific leucine to alanine.

EAR domain i (DLNEPT) and ii (LDLRD) in JAZ5 amino acid sequence are highlighted. Leucine a hydrophobic amino acid was substituted with alanine which is relatively small.

For *JAZ5,* two internal restriction sites (BpiI and BsaI) first had to be removed to proceed with Golden Gate cloning. The "domestication" sites were positioned in the intron so the changes would not affect the final protein sequence. The BpiI restriction site was removed through PCR amplification (Figure 4.4) and digestion into level zero (L0) vector. Once in L0 vector, the BsaI restriction site was removed by Kit based mutagenesis (Figure 4.5). From this point, further mutagenesis using kit based mutagenesis was used to mutate both EAR domains. See Supplementary Data, 5 for a full primer list.

JAZ5 has two EAR motif domains (Figure 4.2 and Supplementary Data). The leucine residue corresponding to the EAR motif sequence, as described by Kagale 2010 (Figure 4.2), was altered to alanine; a basic, nonpolar amino acid (Kagale *et al*., 2010). This was carried out to diminish the repressive nature of the EAR motif and determine whether this altered *JAZ5*s interactions and function during plant pathogen interactions.

AGI Code	Protein Name	Protein Size	EAR-Motif Sequence	EAR-Motif Location
AT1G17380	JAZ5	274	DLNEPT	56
			LDLRL	272

Figure 4.2. JAZ5 conserved EAR motif position and sequence

The location refers to the position of the Leu (L) residue underlined in the corresponding EAR motif sequence.

Figure adapted from Kagale 2010.

Level zero *JAZ5* constructs were constructed in a similar way to *JAZ10*, with the exception that *JAZ5* (1417bp) was amplified in two sections to mutate the internal BpiI site (using primers 31-34); *JAZ5* pro (1557bp). Before proceeding to level one, an internal BsaI site was removed by site-directed mutagenesis (Stratagene) using primer 36 (Figure 4.4 & Supplementary Data section 5). Once the mutation had been confirmed through restriction digestion and sequencing, the construct was used to proceed. eari was subsequently mutated by the same strategy using primer 1. Confirmation of eari (Figure 4.3) mutation was established through sequence alignment. Because earii is positioned at the end of the gene it was possible to mutate using primer 35 and high fidelity PCR. Using *JAZ5* WT and *JAZ5* eari as templates the second EAR motif was mutated using the earii primer, giving *JAZ5* earii mutated and *JAZ5* eari/ii mutated genes. These four *JAZ5* constructs were then assembled into level one vector (see below), this time in position two, which later allowed double constructs to be assembled and sequence validated. The final constructs were;

pGBKT7::BASTA-*JAZ5*$_{pro}$:3xHA:*JAZ5*:Act2

pGBKT7::BASTA-*JAZ5*$_{pro}$:3xHA:*JAZ5*eari:Act2

pGBKT7::BASTA-*JAZ5*$_{pro}$:3xHA:*JAZ5*earii:Act2

pGBKT7::BASTA-*JAZ5*$_{pro}$:3xHA:*JAZ5*eari/ii:Act2

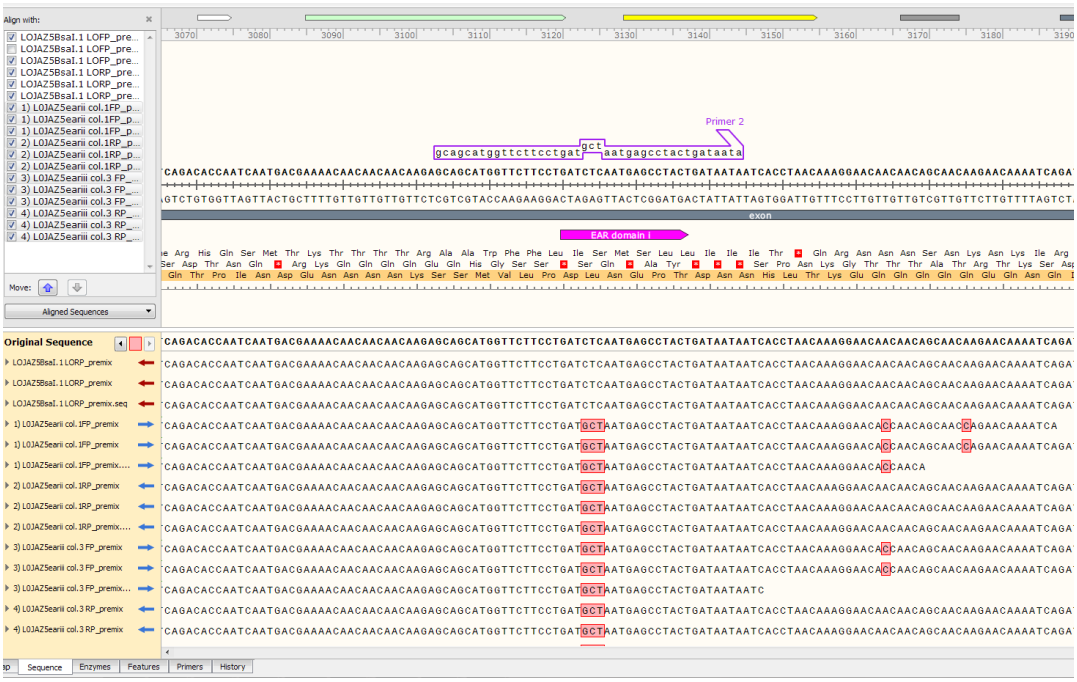


Figure 4.3. Sequence alignment of Level zero JAZ5 eari mutated constructs.

From sequence alignment JAZ5eari domain has been successfully mutated and is present in L0 plasmid. Stocks were made and stored for future use.

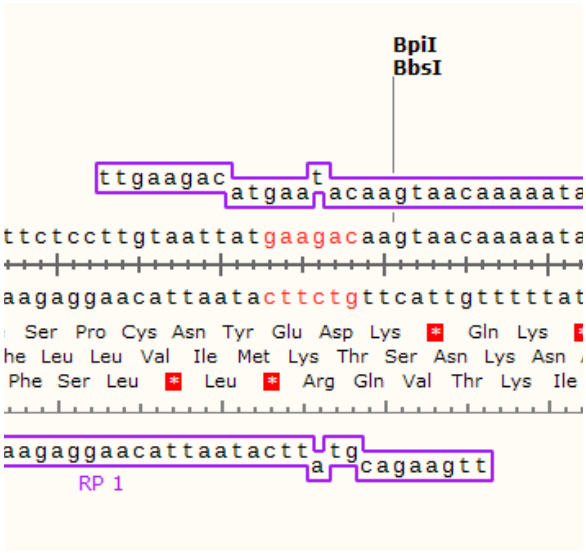


Figure 4.4 L0-JAZ5 gene with internal BpiI restriction site was removed

The *JAZ5* gene was amplified in two sections. Primers over the internal BpiI recognition site cause a substitution removing the enzyme recognition sequence. The primer overhang contains a BpiI recognition site, so during L0 assembly using BpiI, fragment ends were digested and re-ligate together. Sequence alignment was confirmed with expected mutation.

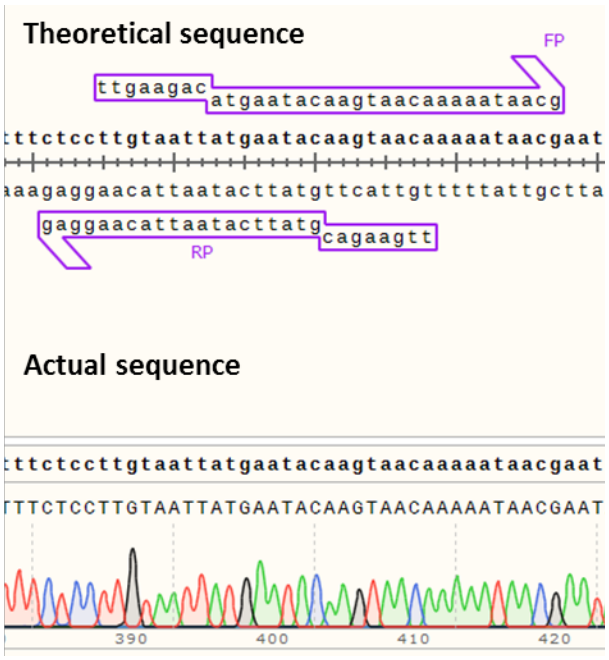


Figure 4.5 L0-JAZ5gene with internal BpiI restriction site removed and sequenced

HF-PRC amplified fragments mutated the internal BpiI recognition site. Fragments were digested with BpiI and ligated back together in a L0 vector. Sequencing across this section indicated mutation and ligation was successful.

Level 1 Position 2 pICH47742 assembly

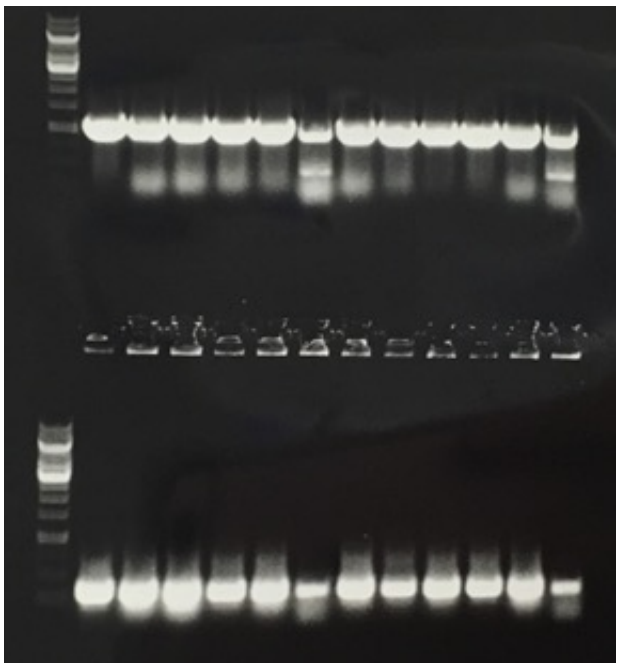


Figure 4.6 JAZ5earii PCR diagnostic
Primer combination, A) L2-4723 FP (plasmid) and NOS promoter RP (on BASTA position one), 946bp; B) *JAZ5* promoter FP and *JAZ5* gene RP, 330bp with HA tag. Twelve colonies were tested, all indicating successful ligation of parts into a vector.

Level zero (LO) *JAZ5* gene WT, eari, and earii were assembled in level one (L1) acceptor vector, pICH4774 with the same stocks of *JAZ5* promoter, Ac2 terminator and HA epitope tag. N terminal PCR diagnostic of positive colonies across the HA tag (*JAZ5* promoter FP and *JAZ5* gene RP) and gene-terminator junction (*JAZ5* gene FP and Act2 RP) were amplified. From the six colonies of each assembly only *JAZ5* earii mutated was positive. Repeat PCR testing of a larger number of colonies (twelve more) confirmed that only *JAZ5*earii could be taken forward (see Figure 4.6). Due to assembly miscarriage the other three were constructed directly into a level two-acceptor vector pICH86966 (see Figure 4.8). L1 **p**osition two (P2) *JAZ5*earii cassette was then successfully assembled into L2 accepter vector with L1 P1 BASTA; pGBKT7::BASTA-*JAZ5*$_{pro}$:3xHA:*JAZ5*earii:Act2. The construct was validated by PCR diagnostics, digestion and sequencing.

L2 pICH86966 - JAZ$_{pro}$:HA:*JAZ5*$_{gene}$:Act.2

Due to unsuccessful level two, position two (L2P2) assembly described above, a different approach using pICH86966 was used. Appropriate L0 constructs were directly ligated into pICH86966, avoiding L1 construction using BsaI enzyme in a one pot digestion and ligation reaction (see Figures 4.7 and 4.8).

pICH86966::*JAZ5*$_{pro}$:3xHA:*JAZ5*:Act2 - kanamycin

pICH86966::*JAZ5*$_{pro}$:3xHA:*JAZ5*eari:Act2 - kanamycin

pICH86966::*JAZ5*$_{pro}$:3xHA:*JAZ5*eari/ii:Act2 - kanamycin

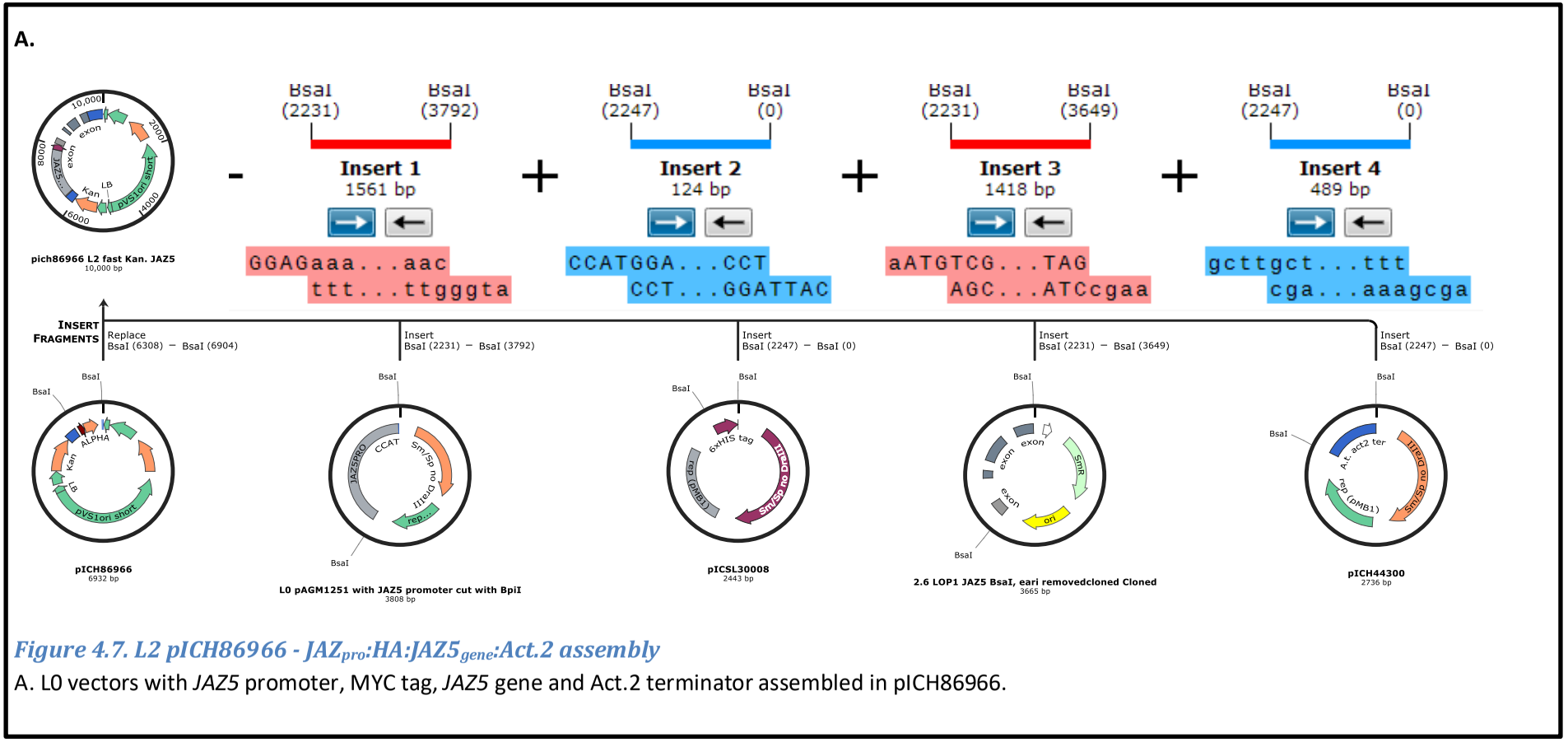


Figure 4.7. L2 pICH86966 - JAZ_{pro}:HA:$JAZ5_{gene}$:Act.2 assembly

A. L0 vectors with *JAZ5* promoter, MYC tag, *JAZ5* gene and Act.2 terminator assembled in pICH86966.

B.

Figure 4.8. L2 pICH86966 - JAZ_{pro}:HA:$JAZ5_{gene}$:Act.2 assembly

B. L2 vector map, total vector size of 10 000 bp. L2 vector has a kanamycin resistance cassette with NOS promoter and terminator for selection.

L2 pICH86966 - JAZ_{pro}:HA:$JAZ5_{gene}$:Act.2 Fast assembly

Due to initial unsuccessful L2P2 assembly for *JAZ5* WT, eari and eari/ii, a different approach using pICH86966 was developed. In this approach, L0 constructs can be ligated directly into pICH86966, avoiding L1 construction. A drawback to this approach is that the resistance cassette, already in the plasmid, is kanamycin. Kanamycin has a lower selection efficiency in plants compared to BASTA and can make the homozygous selection process longer.

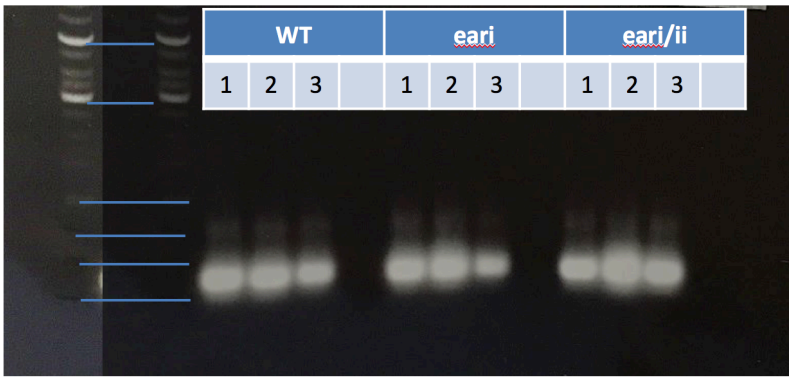


Figure 4.9. L2 pICH86966 - JAZpro:HA:JAZ5gene:Act.2 Fast assembly PCR diagnostics

JAZ5 promoter FP and *JAZ5* gene RP, across the HA tag (194bp), expected amplification 330bp.

Positive colonies selected through PCR diagnostics across the HA tag, Figure 4.9, and left border primer to promoter. Digestion of the plasmid confirmed the expected size. The resulted construct was introduced to *Agrobacterium* competent cells (GV3101) by heat shock transformation. Then later transformed into flowering plants by the floral dip method.

Col-0, *jaz5, jaz10 and jaz5/jaz10* plants were transformed with *Agrobacterium* containing the relevant construct by floral dipping. Seeds were collected. Transformed seeds were then selected on kanamycin antibiotic agar plate or BASTA treated soil as appropriate.

Discussion

This project focusses on the role of transcriptional repression in plant immunity. Specifically it studies two *Arabidopsis thaliana* transcriptional repressors with diverse functions that have been experimentally validated to be targeted by bacterial effectors of the virulent hemi-biotrophic pathogen, *pseudomonas syringae* pv. *tomato* strain DC3000 (Glazebrook, 2005; Grant and Lamb, 2006; Robert-Seilaniantz, Grant and Jones, 2011; Gimenez-Ibanez and Solano, 2013).

Experimental evidence to date shows that these transcriptional repressors target two hormone pathways that are known to be modulated by DC3000 type III effectors, abscisic and jasmonate signalling (Greenberg and Vinatzer, 2003). One, HUB37, is a MYB transcription factor that has diverse functions being recently identified to have roles in hypocotol elongation (Kwon *et al*., 2013; Lu *et al.,* 2014).
The other, JAZ5 is a transcriptional repressor of jasmonate signalling, functioning in conjunction with JAZ10 to attenuate bacterial virulence (de Torres *et al.*, 2015).
Both these immune signalling components have EAR domains, transcriptional repressor domains, notably, JAZ5 has two domains (see Figure 4.1) (Kagale, 2010).
This project set out to further characterise the function of JAZ5 and in particular HUB37 as its role in plant immunity has not been to date reported. HUB37, is so named as it was predicted to be a central HUB in a Bayesian State Space Model generated using gene expression profiles of DC3000 infected tissue over a 13 time point microarray experiment and modelled against known components of ABA signalling and biosynthetic pathways (Lewis *et al*., 2015; S. Jayaraman & M. Grant unpublished results). Strikingly *HUB37* expression is strongly suppressed upon DC3000 infection, suggesting attenuation of HUB37 is necessary for full disease symptoms. Consistent with this, as I arrived in the laboratory, *hub37* KO line was shown to be more resistant to DC3000, though currently the mechanism behind this resistance remains to be elucidated. As

part of the initial work on the project I also looked at whether two other *MYB* genes differentially regulated between virulent DC3000 and non-pathogenic DC3000*hrp* and predicted to represent transcriptional hubs (*HUB23* and *HUB17*) were functionally associated with HUB37. The *hub37/hub17* double mutant showed additional enhanced resistance suggesting these two *MYBs* co-operated in promoting disease development (negative regulators of plant immunity).

The core part of the initial project work involved characterised transgenic lines expressing HA epitope tagged HUB37 in both Col-0 and *hub37* mutant backgrounds (transgenic primary transformants generated by Marta de Torres) under a strong 35S cauliflower mosaic virus promoter.

This construct appeared to complement mutant *hub37*, but strikingly, I was able to demonstrate that HUB37-HA was degraded during disease development caused by DC3000 but not the DC3000hrp mutant (Figure 3.5). This is an exciting result as it is one of the few examples I am aware of, of a plant transcription factor being targeted for degradation by bacterial effectors early in the infection process. This result is somewhat counterintuitive, as the *hub37* mutant is more resistant to DC3000 infection (Figure 1.6) (de Torres, unpublished). This suggests that HUB37 is a negative regulator of plant immunity but the data shows that it disappears during infection. The most consistent hypothesis to explain this, is that HUB37 negatively regulates either susceptibility genes or pathways required to promote pathogen virulence and it's displacement early in the infection process may be mediated by a specific proteolytic degradation, hence the loss of the epitope tag. This was one avenue of research being pursued at the time I decided to write a Masters.

Simultaneously, I had used GoldenGate cloning techniques to generate constructs of *HUB37* with a mutated EAR domain and successfully cloned this variant under its own promoter into a T-DNA expression vector ready for transformation into the *hub37* background.

In parallel to these studies I wanted to extend the novel studies of de Torres *et al*. (2015) who showed the *JAZ5* and *JAZ10* co-operated to confer immunity to DC3000 infection. The JAZ family of transcriptional repressors compromises 12 members and this was the first example showing co-operativity amongst the

JAZ proteins. Importantly, JAZ5 contained 2 EAR domains so I used GoldenGate cloning to generate JAZ5/JAZ10 constructs with both epitope TAGs and either individual, or both JAZ5 EAR domains mutated (Figure, 4.2)(Kagale *et al*,. 2010). These were successfully generated and sequence validated and transformed into the *jaz5/10* background just prior to completing my labwork.

While disappointing to not take these resources forward into a PhD, it has been both extremely exciting and particularly gratifying to generate so many resources and, particularly, show HUB37 is a negative regulator of plant immunity and is targeted, either for specific proteolytic cleavage or non-specific degradation by effectors.

There are a number of future experiments that are obvious to follow on the initial discoveries. One possibility to test in the future is the effect of a range of protease inhibitors on the stability of HUB37. Serine and cysteine protease inhibitors as well as inhibitors of the 26S protease were the initial ones being considered to test prior to prematurely completing this project (Bode, Halitschke and Kessler, 2013; Dielen *et al*., 2010).

In addition I wanted to address the nature of the protease activity as there are some parallels to the caspase cascades initiated during programmed cell death in *C. elegans* and humans (Conradt, Wu and Xue, 2016; Taylor, Cullen and Martin, 2008). To this end I predict that simple *in vitro* mixing experiments of cytoplasmic supernatants from transgenic lines expressing HUB37 with supernatant from DC3000 infected leaves 15 hpi could be a powerful method to develop, assay and dissect the protease activity using targeted fractionation coupled to mass spectrometry. These would be used to both identify cleavage substrates (or products of HUB37 – see above) and unbiased profiling for proteases.

While it has been frustrating to leave this work uncompleted, the resources developed are now being used at Warwick University. Specifically, a PhD student, Sara Abdelsayed is taking over this work, initially focussing on the HUB37 disappearance while at the same time generating homozygous lines form the JAZ constructs. One new avenue she is pursuing, instigated by my work, is cloning the *hub37* promoter into a yeast 1 hybrid bait vector

(Matchmaker® Gold Yeast One-Hybrid Library Screening System User Manual User Manual Matchmaker® Gold Yeast One-Hybrid Library Screening System User Manual, 2012). This will enable them to screen a transcription factor library to attempt to identify the transcription factors that are responsible for the observed suppression of the *HUB37* transcript following DC3000 infection.

Interestingly, another observation to test is a possible link between HUB37 and ABA signalling. Notably, in addition to be predicted as a key hub in ABA signalling the knockout line of *HUB37* was significantly more resistant to DC3000 than the WT Col_0 (Figure, 3.1), but also had a smaller phenotype compared to Col-0, strikingly reminiscent of the *ABA* biosynthetic mutant *aao3* which is also more resistant to DC3000 (de Torres *et al*., 2009).

There are also some technical considerations going forward. The protein level of HUB37 determined in C1-35S_{pro}:MYB:3XHA-NOS lines was quantified in response to infection with virulent and non-virulent DC3000. Notably western blot analysis of T_3 MYB:HA lines showed HUB37 accumulation was not consistent suggesting some lines were not stable after three generations of hygromycin selection and gene silencing may possibly be occurring (Figure 3.2).
Another issue to address with HUB37 (and relating to the above suggestion of looking at possible HUB37 cleavage products upon its disappearance) is that there is a clear ratio difference between protein size (46Kd) and a smaller immunogenic protein band (Figure 3.4). This indicates that as early as 8hpi there is a higher proportion of potentially degraded protein compared to 0hpi. To address this observation and characterise HUB37 integrity during early infection stages, the next step would be to analyse the 8hpi protein extraction using affinity pull down experiments (HA-affinity column). Through these experiments, it would be possible to establish the nature of HUB37 degradation during bacterial infection and identify potential cleavage sites by mass spectrometry.

In line with previous microarray gene expression time-course data DC3000 reduced HUB37 accumulation compared to non-virulent DC3000*hrpA (Lewis et al*., *2015)*. This suggests the effector proteins secreted through the pilus of

DC3000 are the causal agent of both suppression of *HUB37* transcript and directly HUB37 protein levels. Preliminary data (Figure 3.5) suggest effectors may not be not the sole reason for this decrease in abundance of HUB37 as there also appears to be a small decrease in HUB37 abundance when inoculated with DC3000*hrpA*. However, this difference is minimal compared to mock and DC3000 which suggest protein expression is targeted directly by effector proteins and is involved in ETI. Further studies using more quantitative approaches, such as mass spectrometry as discussed above, are needed to determine whether PTI impacts HUB37 abundance.
The initial results indicate that the EAR mutated lines do act like *hub37*. These data support the hypothesis that the EAR motif is the active repressive domain of HUB37 interacting with other transcription factors. However, further analysis is obviously required. Using a *pKUA1::GUS* expression line to study leaf development it was shown that At5g47390 promoter activity was observed on day 12 at the leaf tip and is present throughout the leaf by day 14, but becomes restricted towards the base of the blade at day 17 (Lu *et al*., 2014). This is consistent with strong activity during the leaf expansion period and may account for the reduced stature of *hub37* knockout lines.

The JAZ5 work is less advanced but none the less interesting. It is hypothesised that the two JAZ5 EAR domains contribute to JAZ5's function as a dominant transcriptional repressor. The mutation created and transgenic lines generated will, when selected for homozygosity, help determine the role of these JAZ5 EAR domains, individually and in combination, in plant immunity.

In summary, the tools that have been produced for HUB37 and JAZ5 expression and Ear domain analysis, particularly the transgenic lines (and the accompanying glycerol stocks) provide a great foundation to characterise the role of these diverse EAR domain containing proteins in plant immunity. Moreover, having made the JAZ derivatives using GoldenGate, the option exists to go back and re-generate other constructs as necessary. For example, different epitope tags could be used and other genes could be inserted into final Level 2 construct. Level 0 *JAZ5* gene mutations can be used as templates and amplified into different systems such as yeast two-hybrid for *in vitro* protein-

protein interaction or yeast 1 hybrid as is currently underway at Warwick University.
Further studies using more quantitative approaches and replicated experiments are needed to determine whether PTI/ETS impacts HUB37 abundance. The most obvious approach is to first test a variety of protease inhibitors and inhibitors of the 26S proteasome to see if these can prevent degradation of HUB37 during PTI, as well as undertaking in vitro supernatant mixing studies as described in detail above.

Overall, I feel it is fair to say that this study has made a significant contribution to both identifying a potentially novel role for HUB37 in plant immunity and generating a valuable set of tools to define the role of HUB37 and the HUB37 and JAZ5 EAR domains in contrasting mechanisms that are central to plant immunity.

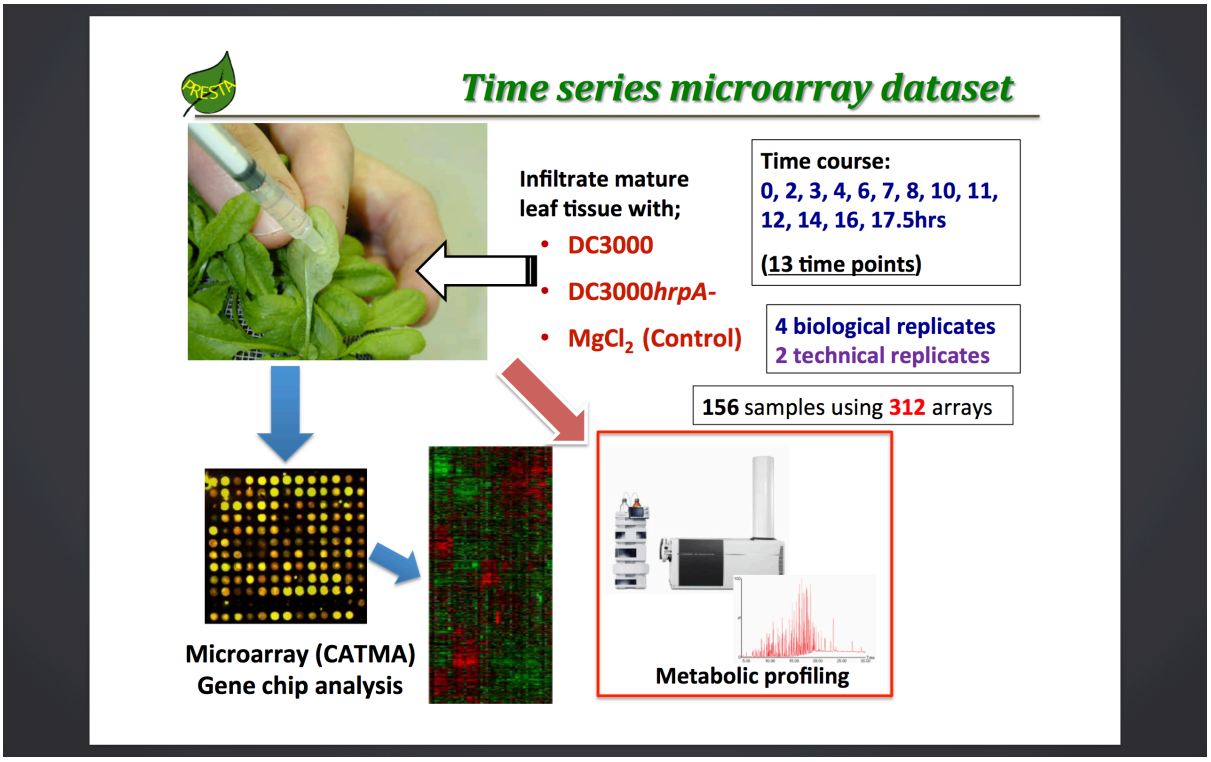
Concluding Remarks

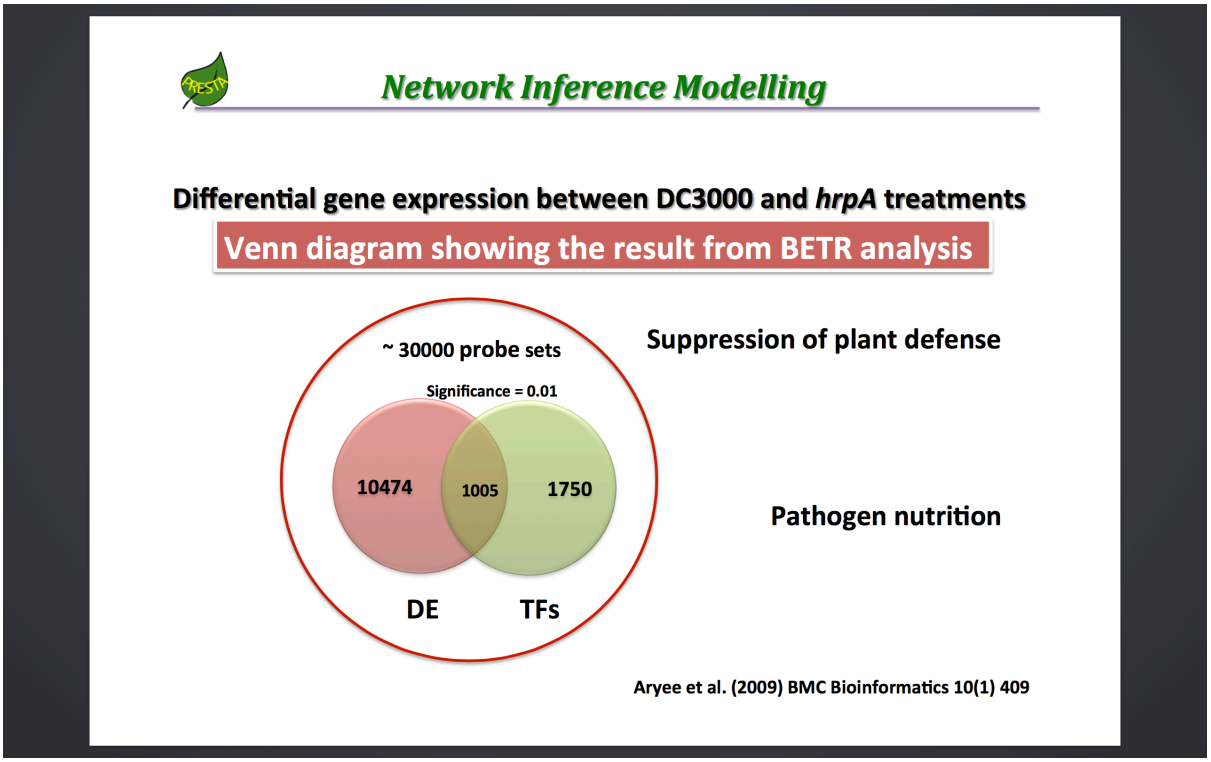
Due to personal circumstances, I decided not to relocate with my research group and therefore could not complete a PhD. However, I have generated an excellent range of tools and validated an interesting phenotype for HUB37 - providing a platform for others to follow up the results I achieved.

Supplementary Data 1 - At5g47390

Supplementary Data 1 - At5g47390 – HUB37

Discovery of Hub37





Slides from Murray Grant

Full length Genomic Sequence

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 101 TCTCTTTCTC ACAATTAGAT TCTGTGCTTC TTCTGCGATC AACTAAGATC
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 201 TCGGGTCGGG TATGACTCGT CGATGTTCTC ACTGCAATCA CAATGGCCAC
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 401 TCTCCGGGTG ATGTCCCTGA CCATGTCGCT GGTGATGGTT ACGCTTCTGA
 451 GGATTTCGTT GCTGGCTCTT CCTCTAGCCG CGAGAGAAAG AAAGGTATCT
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 551 TTGAGTTTAT TTTGCATCAA AGTCGTTGAT TGCATTATGT AACAAGTGGT
 601 GATCTGGTTT ATGTAACAAG ATTTTGATGT GTGTTTGATA TTGGTTTTGT
 651 TGTAGGAACT CCATGGACAG AGGAAGAACA CAGGATGTTC TTATTAGGTT
 701 TACAGAAGCT GGGTAAAGGT GATTGGAGAG GTATCTCAAG AAACTATGTG
 751 ACCACTAGGA CACCTACACA AGTTGCTAGC CATGCTCAGA AGTATTTCAT
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1851 TAGGTTGTTT AACTTGGGTA GGATGTTTTA GTTCAGCTTT GATCATTAGG
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Gene Sequence – EAR domain highlighted

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TGTCCCTGAC CATGTCGCTG GTGATGGTTA CGCTTCTGAG GATTTCGTTG
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CATATTACCC GTTTCCATTC CCAATATGGC CTGCTGGTTA TGTTCCTGAA
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CTCGAAAGCT CCTATCAATG TTGACGAGCT TCTTGGTATG TCTAAGCTCA
GCCTTGCAGA GTCCAACAAA CATGGAGAAT CCGATCAGTC TCTTTCATTG
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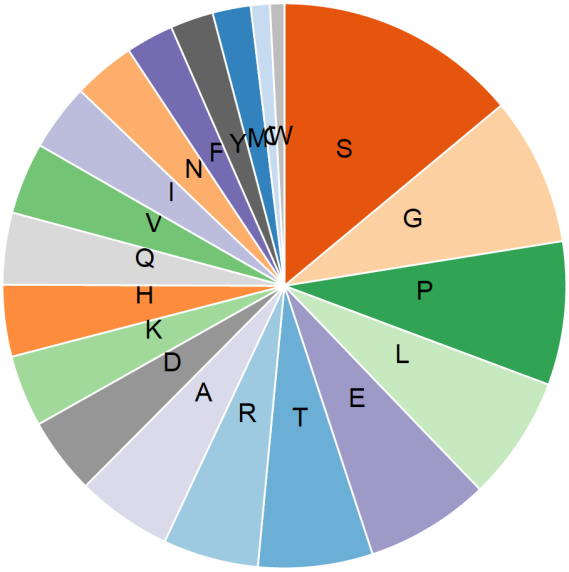
ISI predicted binding sites

Amino Acid Composition



Protein sequence – conserved regions highlighted

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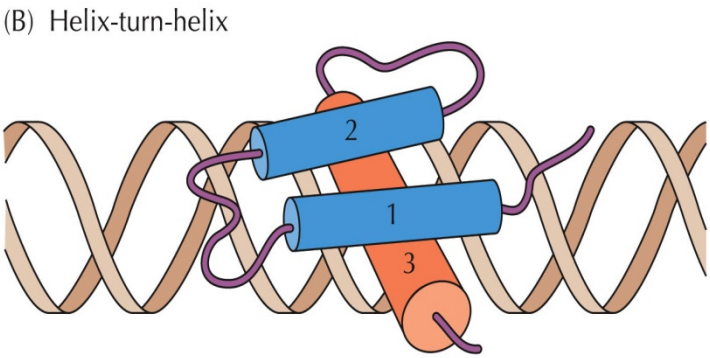
 51  GSGGHGTGSN TPGSPGDVPD HVAGDGYASE DFVAGSSSSR ERKKGTPWTE
101  EEHRMFLLGL QKLGKGDWRG ISRNYVTTRT PTQVASHAQK YFIRQSNVSR
151  RKRRSSLFDM VPDEVGDIPM DLQEPEEDNI PVETEMQGAD SIHQTLAPSS
201  LHAPSILEIE ECESMDSTNS TTGEPTATAA AASSSSRLEE TTQLQSQLQP
251  QPQLPGSFPI LYPTYFSPYY PFPFPIWPAG YVPEPPKKEE THEILRPTAV
301  HSKAPINVDE LLGMSKLSLA ESNKHGESDQ SLSLKLGGGS SSRQSAFHPN
351  PSSDSSDIKS VIHAL
```

At5g47390 contains conserved regions:

- a CCHC-type zinc finger 'RCSHCNH NGHNSRTCPN RG'
  with an R/KLFGV-type repression domain 'KLFGV';
- a R1MYB domain 'PWTE EEHRMFLLGL QKLGKGDWRG ISRNYVTTRT PTQVASHAQK YFIR'
  with a nuclear localization signal 'R ERKK' and 'SRRKRRS';
- a putative leucine-rich nuclear export signal (NES) ' L';
- an EAR-like domain LxLxL (Hiratsu *et al*., 2003) that has the potential to act as a transcriptional repressor domain 'LSLKL';
- and, five low complexity regions.

Schematic diagram of HUB37 Protein

Homology structure of HUB37 biased on TRF2 a human MYB transcription factor (34%). Image from Nature (date accessed 19 January 2016)
http://www.nature.com/ncomms/2014/140507/ncomms4767/extref/ncomms4767-s1.pdf



Supplementary Data 2 - At1g17380

Supplementary Data 2, At1g17380 – JAZ5

Full length Genomic Sequence

(Complementary strand)

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 501 AATAACGAAT AAGTTGATAT AGAAATTGAT TGAGATGTGG TTAATAAAAT
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 651 AGGGCATTCC AAAGGCGAAC CCTCTACCTC ATCAGGAGGC AAAGTCAAAG
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 851 GAAGTTCGCA GCTGACCATA TTCTTCGGAG GGAAAGTTTT AGTATATAAT
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Gene Sequence – EAR domains highlighted

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Protein sequence – EAR domains highlighted

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 151 MVLPDLNEPT DNNHLTKEQQ QQQEQNQIVE RIARRASLHR FFAKRKDRAV
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 251 SDGDKDDIMK IEEGQSSKDL DLRL
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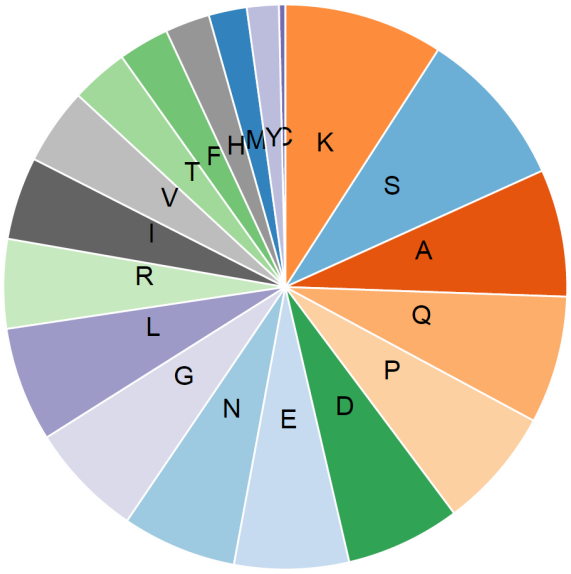
At1g17380.1 contains four conserved domains: (information from http://www.biomedcentral.com/content/pdf/1752-0509-4-43.pdf (date accessed 19 January 2019)

- "TIFY domain - This short possible domain is found in a variety of plant transcription factors that contain GATA domains as well as other motifs. Although previously known as the Zim domain this is now called the tify domain after its most conserved amino acids. TIFY proteins can be further classified into two groups depending on the presence (group I) or absence (group II) of a C2C2-

GATA domain. Functional annotation of these proteins is still poor, but several screens revealed a link between TIFY proteins of group II and jasmonic acid-related stress response.

- Divergent CCT motif - This short CCT_2 motif is found in a number of plant proteins. It appears to be related to the N-terminal half of the CCT motif. The CCT motif is about 45 amino acids long and contains a putative nuclear localisation signal within the second half of the CCT motif.
- EAR domains – transcriptional repressor domain found in plants
- Low complexity region - the genetic mechanisms from which they arise lends them remarkable degrees of compositional plasticity (Coletta, *et al.*, 2010)"

Amino Acid composition



Supplementary Data 3 - Method

Cloning strategy for C1-35Spro:MYB:3XHA-NOS, hemagglutinin sequence

Triple hemagglutinin (HA) sequence from pHB1-HA3: ATG – start codon; GREEN – Protein sequence
DNA sequence:

CC ATG GCA GGT TAC CCA TAC GAC GTT CCT GAC TAT GCG TCA CTC TAC CCC TAT GAC GTA CCG GAT TAT GCA TCC CTA TAT CCG TAT GAT GTT CCA GAT TA C GCT TCT CTA CGT TCC TCT AGA GGC GTC CAC CAT ATG

Protein sequence:

MetAGYPYDVPDYASLYPYDVPDYASLYPYDVPDYASLRSSRGVHH**Met**

Golden Gate Cloning

JAZ5 – EAR Domain Mutagenesis.

Highlighted primer, target change (mismatch) and EAR (Ethylene-Responsive Element Binding Factor-Associated Amphiphilic Repression Motif-Containing Transcriptional Regulators).

AT1G17380.1
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1 MSSSNENAKA QAPEKSDFTR RCSLLSRYLK EKGSFGNIDL GLYRKPDSSL
51 ALPGKFDPPG KQNAMHKAGH SKGEPSTSSG GKVKDVADLS ESQPGSSQLT
101 IFFGGKVLVY NEFPVDKAKE IMEVAKQAKP VTEINIQTPI NDENNNNKSS
151 MVLPDLNEPT DNNHLTKEQQ QQQEQNQIVE RIARRASLHR FFAKRKDRAV
201 ARAPYQVNQN AGHHRYPPKP EIVTGQPLEA GQSSQRPPDN AIGQTMAHIK
251 SDGDKDDIMK IEEGQSSKDL DLRL

Golden Gate plasmids used in Cloning

Rotate Figure on its own page

A Golden Gate Modular Cloning Toolbox for Plants

ACS 2014

Sup 4

sup 2

		Plasmid	Selection	Plate location	
Level 0					
	Clone promoter in	pAGM1251	spectinomycin	E2	level 0 acceptor for pro + 5U(f) modules
Tag vectors	FLAG	pICSL30005	spectinomycin	H6	N terminal FLAG tag (3x FLAG octapeptide)24
	HA	pICSL30008	spectinomycin	A7	N terminal HA tag (6x Human influenza hemagglutinin)25
	Myc	pICSL30009	spectinomycin	B7	N terminal Myc tag (4x Myc)26
	Universal acceptor vector for cloning sequence	pICH41308	spectinomycin	H2	level 0 accepter vector for CDS1 moduels

	Terminator vector	pICH44300	spectinomycin	A12	3'UTR, polyadenylation signal/terminator, act2 (*A. thaliana*) 5
Level 1					
	Level 1 P1 acceptor	pICH47732	carbinicillin	B3	level 1 receptor, position 1, forward orientation
	Level 1 P2 acceptor				level 1 receptor, position 2, forward orientation
					BASTA
Level 2					
	Level 2 acceptor	GBKT7			

Primer List

Box 2014-15	Primer Name	Description of gene	Primer sequence
1	*JAZ5* L156A	*JAZ5*	5'-gcagcatggttcttcctgatgctaatgagcctactgataata-3'
2	*JAZ5* L272A	*JAZ5*	5'-gaagaaggccaaagttcgaaagatctcgatgctaggctatagtaatat-3'
3	jin1-2-FP	*jin1-2*	5'-GACGCTCTGCAGTTTTCTCCACTACGAAG-3'
4	jin1-2-WT-RP	*jin1-2*	5'-CACACCCATGGAACCGATTTTTGAAAT-3'
5	jin1-2-Tr-RP	*jin1-2*	5'-CGAACCATGGTAATAAGGTCCGAACTC-3'
6	B8-jaaz5-FP	*JAZ5*	5'-tggatgaaaattccttttcttcaca-3'
7	B8-jaaz5-RP	*JAZ5*	5'- aggattcaatcttaagaaactttattgc-3'
8	NOS Term FP	*NOS terminator Forward*	5'- TAT GAT AAT CAT CGC AAG ACC GGC AAC AGG -3'

9	NOS Pro RP	*Nos Promoter Reverse*	5'- GTC GCC TAA GGT CAC TAT CAG CTA GCA -3'
10	*JAZ10* FP		5'- AAC CCA TAT CTC TCT GTC TTG A -3'
11	*JAZ10* RP		5'- TCG GAA ACT ACG ACG GCG ATC GA -3'
12	*JAZ5*(b4 stop) FP		5'- GCC TCT TGT ACT CTT CCA TTT TAC GCG C -3'
13	*JAZ5*(after stop) FP		5'- GTG TGG AGA ATT CTT TCT TCT C -3'
14	*JAZ5* RP		5'- CCC TTC TCC TTC AAG TAA CGG C -3'
15	FP *JAZ5* Pro, BpiI		ttgaagacaaGGAGaaaaggacccattcgct
16	RP *JAZ5* Pro, BpiI		ttgaagacaaATGGgttgttgtttattgaga
17	FP *JAZ5* Gene, DraIII		cactctgtggtctcaaATGTCGTCGAGCAATGA
18	RP *JAZ5* Gene, DraIII		cacttcgtggtctcaaagcCTATAGCCTTAGATCGAGAT
19	FP *JAZ10* Pro, BpiI		ttgaagacaaGGAGctacagctcccaacacgtg
20	RP *JAZ10* Pro, BpiI		ttgaagacaaATGGcttctttgatcttattagaaagtgaga
21	FP *JAZ10* Gene, BpiI		ttgaagacaaaATGTCGAAAGCTACCATAGAAC
22	RP *JAZ10* Gene, BpiI		ttgaagacaaAAGCTTAGGCCGATGTCGGAT
23	RP *JAZ5cds EARii*, DraIII		ttcacttcgtggtctcaaagcCTATAGCCTTgcATCGAGAT
24	FP *JAZ5* Gene, DraIII		ttcactctgtggtctcaaATGTCGTCGAGCAATGA

25	RP *JAZ5* Gene, DraIII		ttcacttcgtggtctcaaagcCTATAGCCTTAGATCGAGAT
26	FP *JAZ5* Pro, BpiI		TtgaagacaaGGAGaaaaggacccattcgctACCC
27	RP *JAZ5* Pro, BpiI		ttgaagacaaATGGgttgttgtttattgagaAGAAAG
28	RP *JAZ5cds EARii*, DraIII long		cacttcgtggtctcaaagcCTATAGCCTTgcATCGAGATCTTTCGAACTTTG
29	FP hub37 cDNA, NdeI		gttcaTATGACTCGTCGATGTTCTCACTG
30	RP hub37 cDNA, BamHI		ctgggatccTTATAAAGCGTGTATCACGCTTTTG
31	FP1 *JAZ5*g		ttgaagacaaaATGTCGTCGAGCAATG
32	RP1 *JAZ5*g		ttgaagacaacttattcgttatttttgttacttgtgatcataattacaaggagaa
33	FP2 JAZg		ttgaagacaataagttgatatagaaattgattgagatgtgg
34	RP2 *JAZ5*g		ttgaagacaaaagcCTATAGCCTTAGATCGAGATCTTTCGAACtttg
35	RP3 *JAZ5*ge		ttgaagacaaaagcCTATAGCCTTgcATCGAGATCTTTCGAACtttg
36	*JAZ5* t572a BsaI		gttgctgacctcaggtcactccttttgttccttgg
37	*JAZ10* Exon RP		CAGTGGAAGCTAACAGCGATTTG

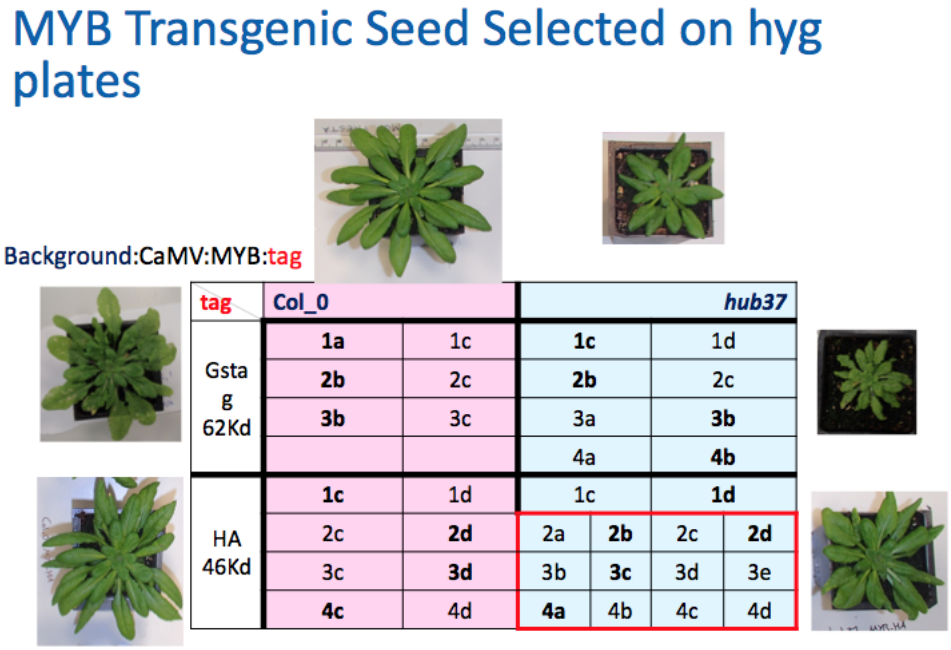
Supplementary Data 4 - Results

Characterise the function of HUB37 in plant defence and selecting homozygotic lines.

To select homozygous complementary and overexpressing HUB37 lines with different epitope tags to identify protein interactions:

–Col_0 and hub37 pC1::35Spro:MYB:3xHA

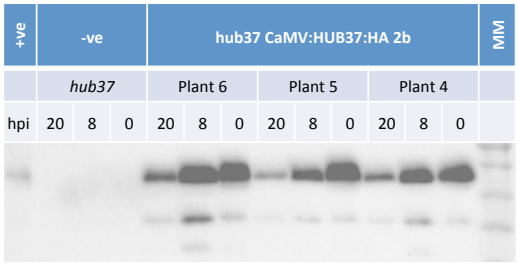
–Col_0 and hub37 pC1::35Spro:MYB:GSTag

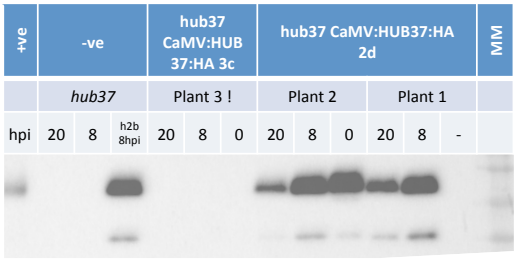


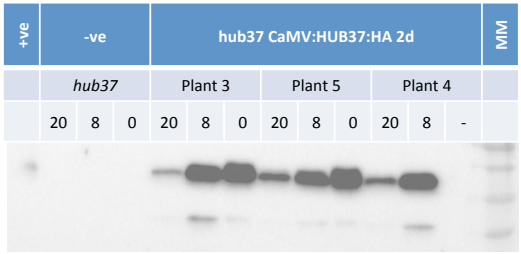
Growth curve and western blot analysis in main text, F*igure 3.3*.

HUB37 Expression Decreases Over Time When Plants are inoculated with DC3000

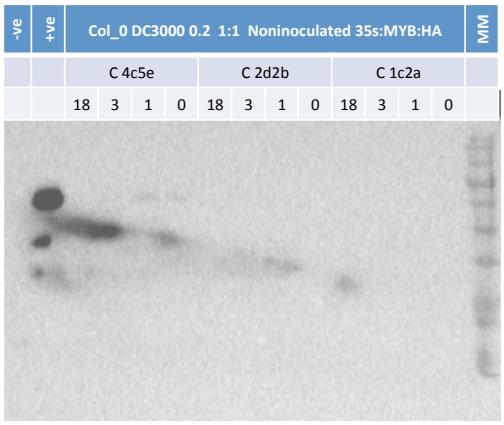
Repeat experiments for HUB37 protein degrading in planta over time when inoculated with DC300.

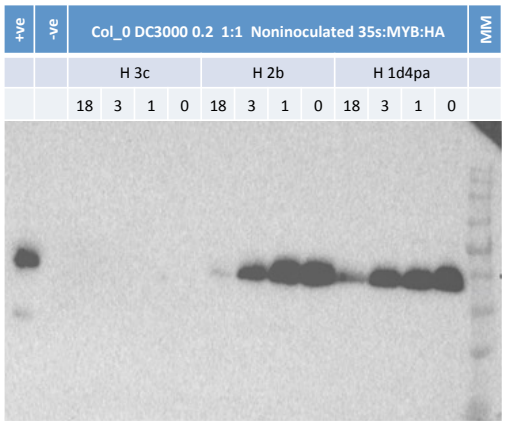


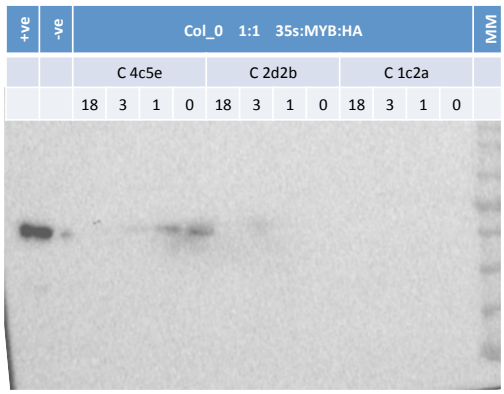




in vitro HUB37 degradation rate







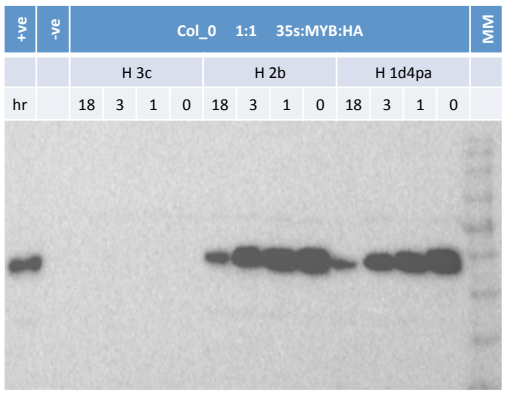


Figure 5.5.1

Degradation of HUB37 *in vitro* over time; Col-0 DC3000 inoculated protein extract was mixed in a one to one ratio with non-inoculated HA:HUB37 expressing lines. Expression of HA:HUB37 too low to visualise in the Col-0 overexpressing background. Clear degradation can be seen in the *hub37* background. Challenged protein mix appeared to degrade more by 18 h than compared to non-inoculated tissue.

Determine if the EAR mofifs in JAZ5 act as the functional repressor.

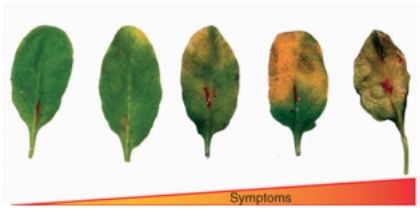
JAZ5 contains both LxLxL and DLNxxP in the C-terminal and middle region respectively.

The repressor activity of the EAR motif found in JAZ proteins is currently unknown

Complementation analysis of JAZ5 and JAZ10 in *jaz5/10* background.

Method

Expression - Plants selected with BASTA (1:500).

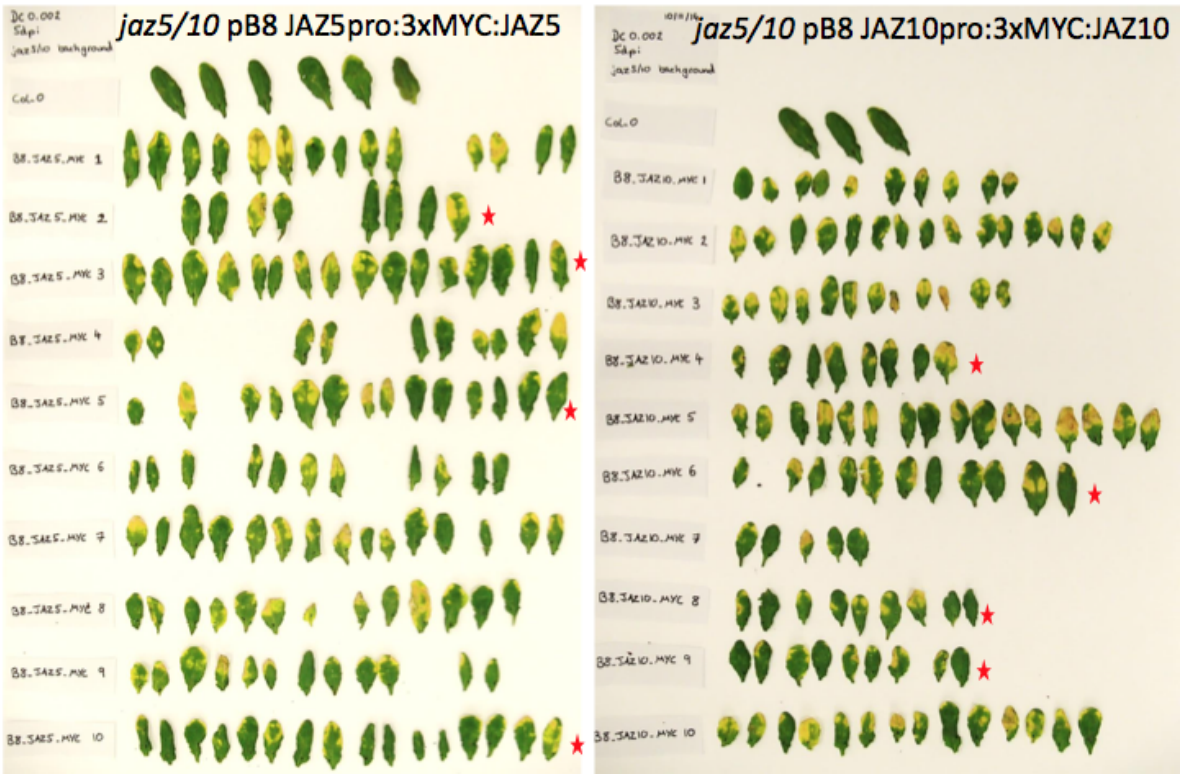


Previous work showed that At-jaz5/10 plants exhibited more chlorosis in comparison to At-jaz10 plants suggesting that JAZ5 and JAZ10 act synergistically.

Function - Two leaves from JAZ5*pro*:3xMYC:JAZ5 and JAZ10*pro*:3xMYC:JAZ10 were inoculated with DC3000: 0.002 for 5 days

- Expect to see:
  - Col_0 — bit chlorotic
  - *jaz5/10* — very chlorotic
  - *jaz5/10* pB8::JAZ5*pro*:MYC:JAZ5 — Similar to Col_0
  - *Jaz5/10* pB8::JAZ10*pro*:MYC:JAZ10 — Even more similar to Col_0

Results: Phenotype



Two leaves per plant were inoculated with PtsD where possible. Some plants died – plants without BASTA resistance which are the gaps. Some every small so only one leaf possible to inoculate.

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